nature portfolio

# Peer Review File

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

Corresponding Author: Dr Yaling Han

**This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.**

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This is a well-designed study with supporting data and promising findings. The study investigated the impact of S100A12 on neutrophil extracellular trap (NET) formation and myocardial injury following acute myocardial infarction (AMI). The findings revealed that S100A12 expression increased rapidly in neutrophils after AMI, leading to enhanced NET production and cardiomyocyte apoptosis, exacerbating myocardial injury. It was demonstrated that S100A12 interacts with Annexin A5 (ANXA5) to enhance calcium influx, promoting NET formation. Blockage of ANXA5 attenuated heart function impairment after MI. Additionally, it was found that hypoxia-inducible factor-1α (HIF1α) directly bound to the S100A12 promoter, increasing S100A12 expression. Plasma S100A12 levels correlated with dsDNA concentration, indicating an increased risk of all-cause mortality after AMI. Overall, these findings highlight the critical role of S100A12 in regulating neutrophil activation and NET formation, suggesting it as a potential marker and therapeutic target for AMI treatment.

Further clarification on the specific role of S100A12 in the context of acute myocardial infarction (AMI) is needed. While the introduction outlines S100A12 as a potential biomarker for AMI diagnosis, it does not delve deeply into its mechanistic involvement in AMI pathophysiology.

Regarding the analysis of cardiac function on day 28 post-procedure, the rationale for this specific time point and whether other time points were considered for analysis should be provided. Additionally, it would be beneficial to know if day 28 was specifically chosen for optimization purposes or if other factors influenced this decision.

Figure 1, legends shows the IHC and the images of the IHC panel is not correctly labeled.

Figure 2 and related results: Did you test the kinetics of neutrophils, NETs, and other types of cell death (such as apoptosis or necrosis) in correlation with S100A12 expression, as understanding neutrophil kinetics in relation to S100A12 is important for comprehensive analysis?

It would be valuable to investigate the mechanisms and types of NETosis occurring in correlation with S100A12. Additionally, the authors should provide evidence of S100A12 promoting NETosis in ex vivo or in vitro experiments. These experiments would enhance our understanding of how S100A12 induces NETosis in neutrophils. Furthermore, it is important to inquire whether other immune cells, such as monocytes and macrophages, were also examined.
Though author tested the levels of MPO and CitH3 but would be worth checking the ROS (either NOX or Mitochondrial ROS) involved in this process of NET formation.

Did the authors check the fate of NETosed neutrophils in terms of apoptosis? As I initially suggested, it is important to test the kinetics of neutrophils/NETs at different time points post-MI for both NETs and apoptosis.
Treatment of DNAse injection was given onward Day7, but the lifecycle of neutrophils and NETs is not that long (Neutrophils infiltration, NETs formations and clearance happened within 48 day), we have observed this in lungs neutrophil peak start from 18 hours and peaked after 32 and last up to 72 hours. So how these time points have been determined. Its always better to have 60X zoomed image of NETs to really see the NETs and colocalization of NETs marker (MPO, CitH3 and NE).

Reviewer #2

(Remarks to the Author)
The manuscript by Zhang et al. describes a mechanistic inside of neutrophils-induced injury after myocardial infarction, which could have significant therapeutical consequences. Currently, we know that neutrophils have controversial roles in healing after myocardial infarction, and despite the convincing experimental results, the translation to clinic have failed. This is due to the current knowledge that neutrophils have a significant contribution to the proper healing and scar formation. Therefore, the scientific world struggle now to identify and dissect between the positive and negative effects of neutrophils, to be able to efficiently select therapeutical targets for clinical translation. The authors describe S100A12 as one of these targets, being responsible in part of the neutrophil's negative effects. The manuscript is well written and convincing, however, there are some comments which should be addressed:
1. It is surprising that S100A12 is expressed in the mice until 4 weeks after myocardial infarction. Since the results that S100A12 can be found in neutrophils 1 day after myocardial infarction are convincing, which cells express S100A12 after 1week, when no neutrophils should be in the affected area? AN overview image showing the presence of S100A12 at the border will be useful.
2. Can DNase I treatment reduce the infarction size and preserve the heart function after administrated to the TG mice? This is an important fact, since S100A12 can be found at later time points after myocardial infarction and can have secondary effects which can annulate the initial positive effect on inflammation (for example on fibrosis and remodeling).
3. Early after myocardial infarction, the proliferation and differentiation of fibroblasts is a crucial factor for proper healing and later scar formation. Since the presence S100A12 induce apoptosis in surrounding cells, what is the effect of the presence of S100A12 in neutrophils on proliferation and differentiation of fibroblasts?
4. The mechanism of S100A12/Annexin5 through calcium-channels and use of calcium-channel blockers is a very interesting and important finding, with direct clinical translation potential, despite the positive effects with the AAV-shANXA5. Can treatment with Verapamil in TG mice reverse the effect of S100A12 expression on cardiac function and infarction size?
5. It is possible to follow if patients within lowest tertile have calcium-blockers in their medication?
Minor comment:
The dual role of neutrophils in healing after myocardial infarction should be highlighted and comment in the manuscript.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This is well laid study and authors have incorporated most of the suggestions. There are few suggestions to improve (optional) or can be discussed in discussion section.
1-Examining how S100A12 levels change over a more extended period could provide a better understanding of its dynamics and whether it has prolonged or delayed effects on NET formation and myocardial injury. Understanding/discussing these temporal patterns could clarify whether S100A12 plays a sustained role in post-MI inflammation or whether its effects are primarily early-stage.

2- Furthermore, a closer look into the specific mechanism of S100A12's interaction with Annexin A5 (ANXA5) to enhance calcium influx may reveal more detailed insights into the signaling pathway involved. Or exploring whether other proteins or signaling molecules play intermediary roles in this interaction might reveal potential targets for therapeutic intervention within the pathway.

3-Therapeutically, assessing pharmacologic inhibitors that target the S100A12 pathway or the S100A12-ANXA5 interaction could provide valuable translational insights. This can be done further by pathway and signaling analysis.

Reviewer #3

(Remarks to the Author)
Thank you for undressing all comments so carefully, I have no additional comments.

**REVIEWER COMMENTS**

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Reviewer #2 (Remarks to the Author):

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Minor comment:

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_____

**RESPONSES TO THE COMMENTS OF REVIEWER #1:**

***Q1***. *Further clarification on the specific role of S100A12 in the context of acute myocardial infarction (AMI) is needed. While the introduction outlines S100A12 as a potential biomarker for AMI diagnosis, it does not delve deeply into its mechanistic involvement in AMI pathophysiology*.

**A1:** We Thank you for your comment. We have revised the paragraph in the manuscript (page 5, line 9) as follows:

S100A12, a newly discovered member of the S100 family of calcium regulatory proteins, is located within the same gene cluster on chromosome 1q21 as other S100A family members. In humans, S100A12 is mainly secreted by neutrophils and monocytes, but can also be expressed by tissue cells, such as mucosal epithelial cells and keratinocytes. Rodents do not express S100A12. The upregulation of S100A12 is primarily driven by cellular stress, which induces its release from granulocytes and other myeloid cells. Upon release, S100A12 binds to its canonical receptor, RAGE, which engages the extracellular domain of this target protein via calcium-bound S100A12. Cumulative studies have demonstrated that S100A12 is associated with many inflammatory, metabolic diseases and cardiovascular events in atherosclerotic diseases. Based on analyses in multivariate Cox proportional hazard models, the S100A12 protein level in peripheral blood has been identified as an independent predictor of major adverse cardiovascular events in patients with stable coronary artery disease. Our previous study revealed that S100A12 is a potential novel biomarker for the early diagnosis and prediction of ST-segment elevation myocardial infarction (STEMI). Recent single-cell sequencing studies showed that S100A12 may serve as a biomarker for neutrophil screening, and can be used for the isolation and analysis of neutrophil subpopulations. However, it is unclear whether S100A12 contributes to the occurrence and prognosis of AMI through the regulation of neutrophils.

***Q2.*** *Regarding the analysis of cardiac function on day 28 post-procedure, the rationale for this specific time point and whether other time points were considered for analysis should be provided. Additionally, it would be beneficial to know if day 28 was specifically chosen for optimization*

*purposes or if other factors influenced this decision.*

**A2:** We thank the reviewer for pointing this out.

Permanent ligation of the left coronary artery is the most commonly used murine model of acute myocardial infarction (AMI) and it imitates the post-MI pathophysiology and immunology in patients. As reported in numerous studies, the pathological process of cardiac tissue, from acute inflammation to chronic repair, was observed from days 1 to 28 after MI in a mouse model. Neutrophilic infiltration of the infarct margin was investigated on days 1 and 2. Marked macrophage infiltration was observed on Day 4. Massive proliferation of fibroblasts and collagen accumulation began on days 7–14, and scar formation was completed by Day 21[1]. One month later, necrotic cardiomyocytes were entirely replaced with fibrotic tissues[2]. The left ventricular (LV) diastolic dimension increased markedly on Day 14 and remained unchanged thereafter. The LV-shortening fraction decreased significantly on Day 14, and gradually decreased thereafter[1]. Therefore, we chose Day 28 as the timepoint of the stable period for evaluating post-MI cardiac dysfunction.

To elucidate the role of S100A12 in post-MI cardiac dysfunction, we established MI models using S100A12 TG mice and their littermates (WT). We assessed the level of ventricular remodeling at post-MI days 7, 14, and 28. Compared with the baseline, cardiac function underwent a sustained decline from post-MI days 7–14. The cardiac function of WT mice on Post-MI Day 28 showed no significant change compared to that on Post-MI Day 14. However, compared to WT mice, TG mice showed no significant difference in cardiac function on post-MI days 7–14. On Post-MI Day 28, cardiac function significantly decreased in the TG group. These results are similar to the pathological damage during MI that has been reported in previous studies, and suggest that S100A12 may exacerbate the chronic damage in post-MI cardiac function in mice (**Figure to reviewer 1**).

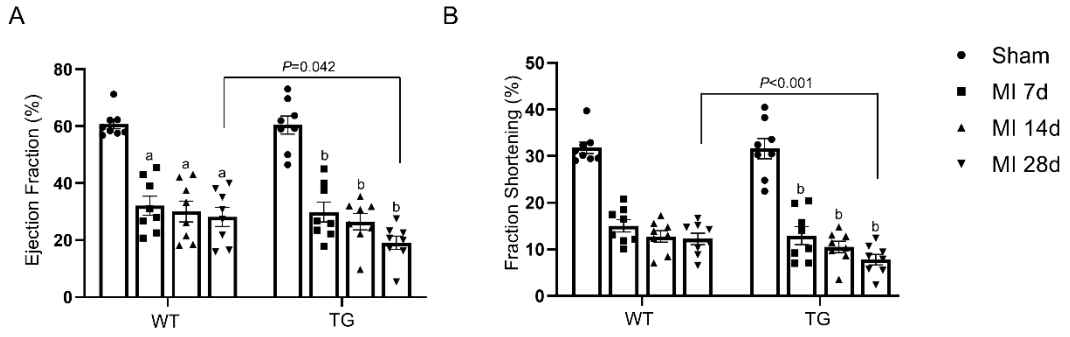



**Figure to reviewer 1. Echocardiographic parameters of WT and TG mice at different post-MI timepoints.**

Data are expressed as mean ± SEM. a: $P < 0.001$ vs WT Sham. b: $P < 0.001$ vs TG Sham.

*Q3. Figure 1, legends show the IHC and the images of the IHC panel is not correctly labeled.*

**A3:** We thank the reviewer for pointing out this problem.

The section of "immunohistochemistry (IHC) staining…" in the legend of Figure 1 was to be deleted, but was carelessly overlooked. We corrected this error in the revised manuscript.

*Q4. Figure 2 and related results: Did you test the kinetics of neutrophils, NETs, and other types of cell death (such as apoptosis or necrosis) in correlation with S100A12 expression, as understanding neutrophil kinetics in relation to S100A12 is important for comprehensive analysis.*

**A4:** Thank you for your comment. As you have pointed out, neutrophil kinetics play a crucial role in establishing the inflammatory response to injury. To elucidate whether S100A12 participates in the pathogenesis of MI through the regulation of neutrophil kinetics, we tracked neutrophils spatially and temporally *in vivo* in S100A12 TG and WT groups with or without MI. Using flow cytometry, we determined neutrophil counts and the ratios of mature neutrophils and granulocyte–monocyte progenitors (GMP) in the blood and bone marrow (BM) of WT and TG mice with or without MI[3]**.** We found that the total neutrophil counts in the BM and blood did not differ significantly between the WT and TG sham groups. On Post-MI Day 1, compared to WT mice, TG mice had a significantly higher neutrophil count in the peripheral blood and a decreased neutrophil count in the BM (**Figure to reviewer 2A** and **2B**). Ly6G expression increased rapidly, peaked on post-MI Day 1, and progressively decreased in the infarct margin in the cardiac tissue of TG mice (**Figure to reviewer 2C** and **2D**). These data suggest the more potent ability of S100A12 to mobilize BM neutrophils into the peripheral blood.

Next, we found that, compared to WT mice, with or without MI, TG mice had a significantly lower percentage (**Figure to reviewer 2F–2G)** and absolute number (**Figure to reviewer 2H–2I**) of mature neutrophils ($CD45^+$ $Ly6G^+$ $CD101^+$) in both the blood and BM. Subsequently, through flow cytometry, in the BM, the GMP population was identified as $Lineage^-$ $c\text{-}Kit^+$ $Sca\text{-}1^-$ $CD16/32^+$ $CD34^+$ cells (**Figure to reviewer 2K**). We observed a trend towards a higher percentage and cell counts of GMP in the BM of TG mice compared with WT mice in both the sham and MI groups (**Figure to reviewer 2L** and **2M**). Collectively, the increased percentage of GMP in the BM and more immature neutrophils in the BM and blood suggest that emergency granulopoiesis-like conditions were induced following S100A12 overexpression. These data suggest that S100A12 regulates neutrophil kinetics and may be involved in MI. We have added these results to the Discussion section of the revised manuscript (page 19, line 1).

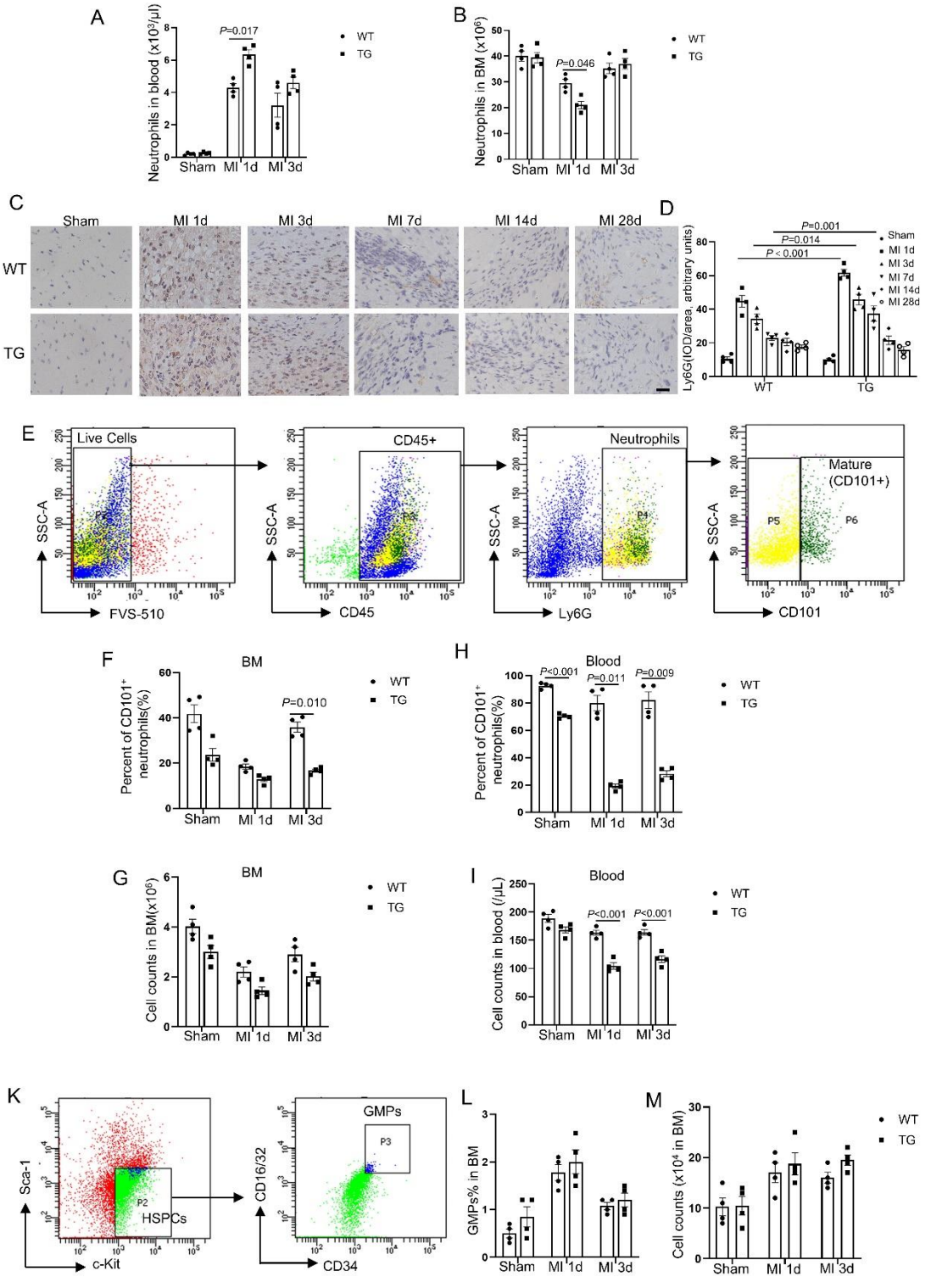
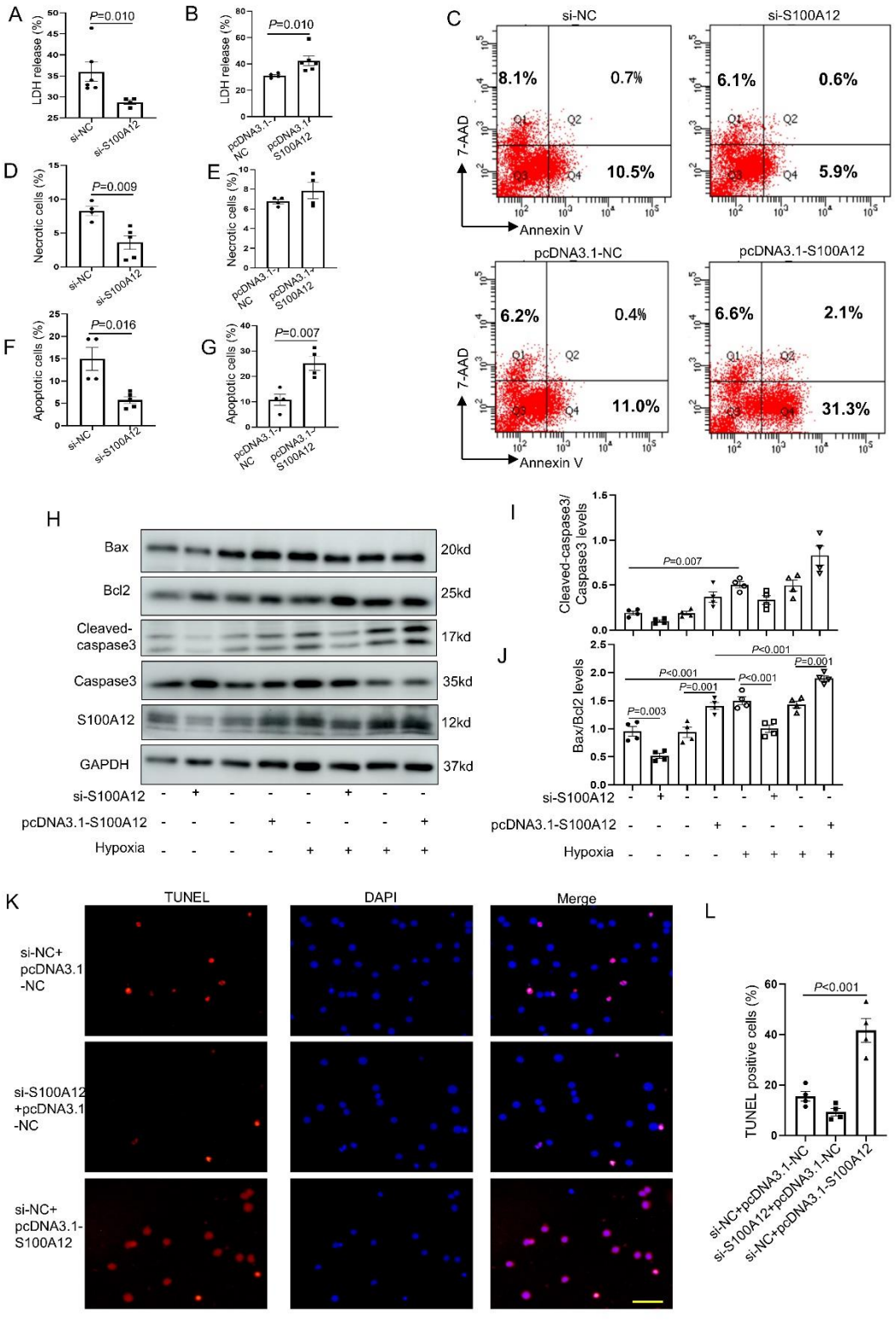



**Figure to reviewer 2. S100A12 expression altered neutrophil kinetics in mice.**

(A) WT and TG mice were subjected to MI by permanent ligation of the left anterior descending artery and their blood neutrophils were quantified on post-MI days 1 and 3. (B) MI-induced changes in the neutrophil count in the BM (cells pooled from two femurs and two tibias). (C, D) Representative IHC staining of Ly6G and quantification in the margin of the infarcted myocardial

tissue of WT and TG mice at different post-MI timepoints. Scale bar, 20 μm. (E) Representative flow cytometry dot plots and a gating strategy used to identify mature neutrophils ($CD45^+$ $Ly6G^+$ $CD101^+$) in the BM. A similar gating strategy was used to identify mature neutrophils in the blood. (F–I) Percentages and absolute cell counts of mature neutrophils in the bone marrow and blood on post-MI days 1 and 3. (K) Representative flow cytometry dot plots and a gating strategy used to identify mature GMPs ($Lineage^-$ c-$Kit^+$ Sca-$1^-$ $CD16/32^+$ $CD34^+$) in the BM. (L) Quantifications of GMPs as a percentage of total live single cells from BM on post-MI days 1 and 3. (M) Absolute GMP counts in the BM were obtained by multiplying GMP percentage with total cell counts. Data are expressed as mean ± SEM.

To explore the relationship between S100A12 and neutrophil death, we measured the activity of lactate dehydrogenase (LDH), a biomarker of necrotic cell death, in the supernatants of cultured neutrophils transfected with si-S100A12 or pcDNA3.1-S100A12. The results showed that S100A12-overexpressing neutrophils exhibited a significant increase in LDH release compared to cells with low S100A12 expression (**Figure to reviewer 3A** and **3B**). The types of cell death were detected using flow cytometry with 7-AAD and Annexin V staining. Cells in the 7-AAD-positive and Annexin V-negative regions are commonly recognized as necrotic cells, whereas 7-AAD-negative and Annexin V-positive cells are recognized as apoptotic cells. We observed that the proportions of both apoptotic and necrotic cells were elevated in S100A12-overexpressing neutrophils, and that the inhibition of S100A12 downregulated the proportions of apoptotic and necrotic cells (**Figure to reviewer 3C–3G**). Western blotting indicated that S100A12 expression closely correlated with the level of apoptosis in neutrophils (**Figure to reviewer 3H–3J**). TdT-mediated dUTP Nick-End Labelling (TUNEL) staining demonstrated that transfection of si-S100A12 reduced the level of apoptosis in neutrophils whereas transfection of pcDNA3.1-S100A12 significantly increased the level of apoptosis (**Figure to reviewer 3K** and **3L**). This suggests that S100A12 significantly increases apoptosis in neutrophils. Consistent with this, we detected the expression of cleaved-caspase 3 and Ly6G at the margin of the infarcted tissue by IF co-staining in WT and TG MI mice. Consistent with the *in vitro* results, S100A12 contributed to post-MI neutrophil apoptosis (**Figure to reviewer 3M** and **3O**).


A
LDH release (%)
P=0.010
si-NC
si-S100A12
B
LDH release (%)
P=0.010
pcDNA3.1-NC
pcDNA3.1-S100A12
C
si-NC
si-S100A12
8.1%
0.7%
10.5%
6.1%
0.6%
5.9%
7-AAD
Annexin V
pcDNA3.1-NC
pcDNA3.1-S100A12
6.2%
0.4%
11.0%
6.6%
2.1%
31.3%
D
Necrotic cells (%)
P=0.009
E
Necrotic cells (%)
F
Apoptotic cells (%)
P=0.016
G
Apoptotic cells (%)
P=0.007
H
Bax
Bcl2
Cleaved-caspase3
Caspase3
S100A12
GAPDH
20kd
25kd
17kd
35kd
12kd
37kd
si-S100A12
pcDNA3.1-S100A12
Hypoxia
I
Cleaved-caspase3/Caspase3 levels
P=0.007
J
Bax/Bcl2 levels
P=0.003
P<0.001
P=0.001
P<0.001
P<0.001
P=0.001
si-S100A12
pcDNA3.1-S100A12
Hypoxia
K
TUNEL
DAPI
Merge
si-NC+pcDNA3.1-NC
si-S100A12+pcDNA3.1-NC
si-NC+pcDNA3.1-S100A12
L
TUNEL positive cells (%)
P<0.001
si-NC+pcDNA3.1-NC
si-S100A12+pcDNA3.1-NC
si-NC+pcDNA3.1-S100A12

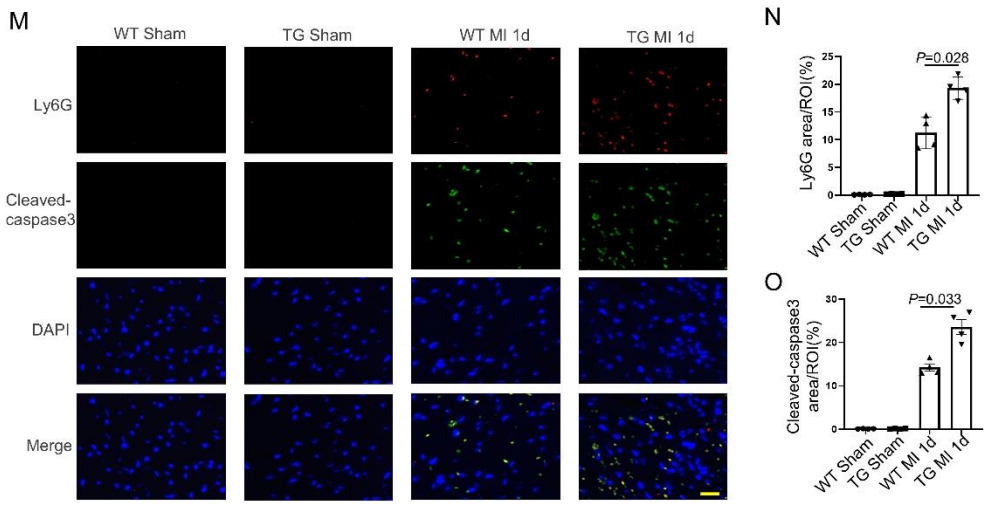



**Figure to reviewer 3. S100A12 expression increased neutrophil apoptosis/necrosis.**

(A, B) Lactate dehydrogenase (LDH) release was detected from the supernatant of neutrophils. (C) Representative flow cytometry images of neutrophils transfected with si-S100A12 or pc3.1-S100A12. (D–G) Percentages of necrotic (Annexin $V^-7AAD^+$) and apoptotic (Annexin $V^+7AAD^-$) neutrophils. (H–J) dHL-60 cells were transfected with pcDNA3.1-S100A12 or si-S100A12 under normoxic or hypoxic conditions. Western blotting was conducted to detect the protein expression ratios of cleaved-caspase3/Caspase3 and Bax/Bcl2. (K-L) Representative images of TdT-mediated dUTP nick-end Labelling (TUNEL) staining and analysis of apoptosis rates in dHL-60 cells transfected with si-S100A12 or pcDNA3.1-S100A12. Scale bar, 50 μm. (M–O) Immunofluorescence double staining of the neutrophil marker (Ly6G) and cleaved-caspase 3 at the infarct margin of TG and WT mice and quantification on Post-MI Day 1. Scale bar, 20 μm. Data are expressed as mean ± SEM.

***Q5****: It would be valuable to investigate the mechanisms and types of NETosis occurring in correlation with S100A12. Additionally, the authors should provide evidence of S100A12 promoting NETosis in ex vivo or in vitro experiments. These experiments would enhance our understanding of how S100A12 induces NETosis in neutrophils. Furthermore, it is important to inquire whether other immune cells, such as monocytes and macrophages, were also examined. Though author tested the levels of MPO and CitH3 but would be worth checking the ROS (either NOX or Mitochondrial ROS) involved in this process of NET formation.*

**A5**: Thank you for your comment. To better understand and answer your questions, we have divided it into 3 separate questions.

***Q5.1****-The authors should provide evidence of S100A12 promoting NETosis in ex vivo or in vitro*

*experiments. These experiments would enhance our understanding of how S100A12 induces NETosis in neutrophils*.

**A5.1**: Thank you for the comment. The results of the *ex vivo* study have been described in our manuscript and specify how S100A12 promotes NETs production in primary neutrophils isolated from WT and TG mice (**Figure 3A–3F** in the manuscript). In the *in vitro* study, we retransfected dHL-60 cells with pcDNA3.1-S100A12 or pcDNA3.1-NC, and SYTOX Green staining was performed 6 h after transfection. NETs production was observed in the live cell imaging system shown in **Figure to reviewer 4**.

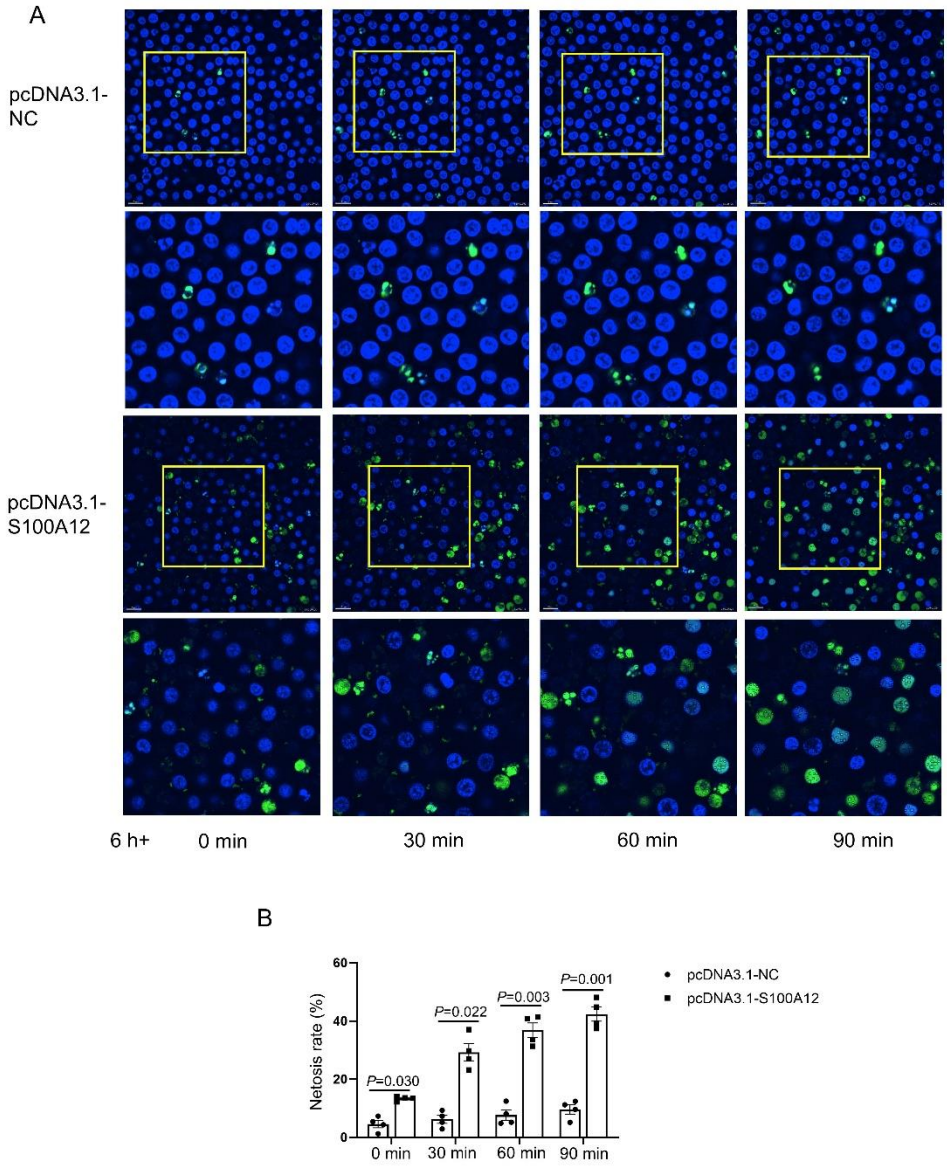


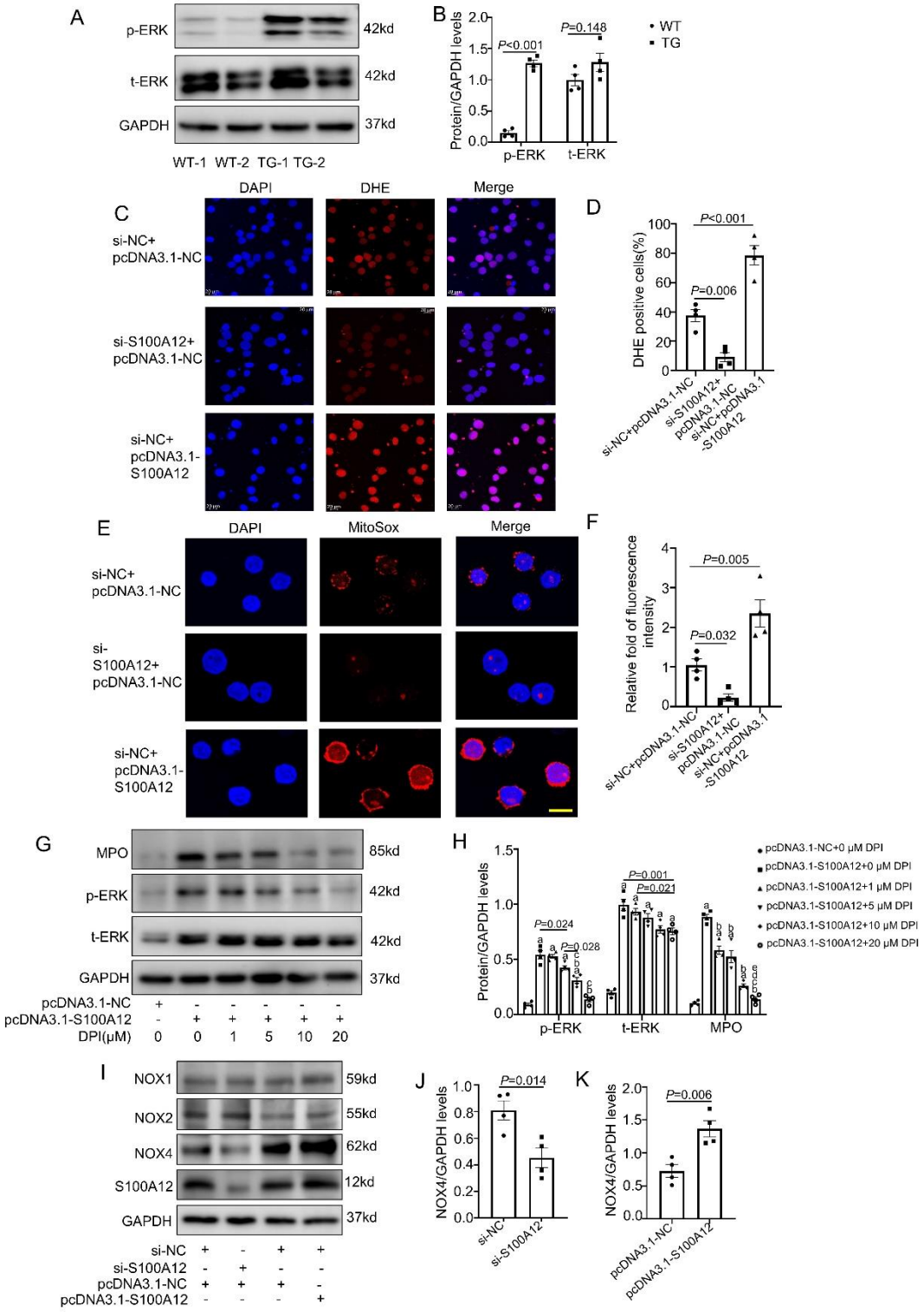

**Figure to reviewer 4. *In vitro* NET formation monitored using live cell imaging system**. Six hours after transfection with pcDNA3.1-S100A12, the dHL-60 cells were stained with SYTOX Green and Hoechst and monitored using a live-cell imaging system. Cells were

photographed every 5 min for 300 min. Important timepoints are shown in minutes. Scale bar, 15 μm.

*Q5.2-It would be valuable to investigate the mechanisms and types of NETosis occurring in correlation with S100A12. Though author tested the levels of MPO and CitH3 but would be worth checking the ROS (either NOX or Mitochondrial ROS) involved in this process of NET formation.*

**A5.2:** Thank you for the comment. Based on the reviewer's suggestion, we reviewed the relevant literature. Three models of NETosis have been established. (a) Suicidal NETosis, with a duration of 2–4 hours, is the best-described model. Stimulation of neutrophils with phorbol 12-myristate 13-acetate activates NADPH oxidase (NOX) via protein kinase C and rapidly activates the fibrosarcoma (Raf)-MAPKP/ERK kinase (MEK)-ERK signaling pathway[4]. NOX activation induces reactive oxygen species (ROS) generation and increases calcium influx[5], with the subsequent activation of arginine deiminase 4 (PAD4). (b) In vital NETosis with nuclear DNA release, neutrophils release NETs within 5–60 minutes without exhibiting loss of the nuclear or plasma membrane, and this is independent of the ROS and Raf/MEK/ERK pathways[5]. (c) The final type is vital NETosis involves the release of mitochondrial DNA, is ROS-dependent, and is induced by stimulation with GM-CSF and lipopolysaccharide.

As S100A12 induced NETosis is a sterile inflammation, we examined whether suicidal NETosis occurred. First, we observed that the p-ERK/t-ERK ratio was higher in primary neutrophils derived from TG mice than in those derived from WT mice (**Figure to reviewer 5A** and **5B**). This result suggests that S100A12-induced NETosis is primarily suicidal NETosis. As suggested by the reviewer, NET induction depends on ROS, for which the main source is NOX. Activation of NOX depends on an increase in the cytoplasmic concentration of $Ca^{2+}$ and, in some cases, on mitochondrial ROS generation. We observed that S100A12 overexpression in dHL-60 cells was associated with enhanced superoxide production, as detected by dihydroethidium (DHE) and MitoSOX Red staining. In contrast, S100A12 silencing reduced superoxide production (**Figure to reviewer 5C–5F**). The addition of the ROS inhibitor diphenyleineiodonium (DPI) dose-dependently reduced NET formation in dHL-60 cells transfected with pcDNA3.1-S100A12 (**Figure to reviewer 5G** and **5H**). Furthermore, we examined the correlation between several NOX isoforms and S100A12 expression and found that S100A12 correlated more closely with NOX4 (**Figure to reviewer 5I–5K**). Among the seven mammalian NOXs (NOX1-5 and DUOX1-2), NOX4 has the most widespread tissue distribution and mediates protective effects during cellular stress. Furthermore, an ATP-binding motif within NOX4 functions as a mitochondrial energy sensor that inhibits NOX4-derived ROS production and thereby promotes pro-survival pathways in renal carcinoma cells[6]. These properties render NOX4 ideal for linking mitochondrial bioenergetic failure to cell death. We have added the pertinent results to the Results and Discussion sections of the manuscript as **Supplemental Figure 6** (page 12, line 6-18, and page 18, line 14-15;).




**Supplemental Figure 6-Figure to reviewer 5. Endogenous S100A12 promotes suicide NETosis by the NOX4-ROS-pERK pathway.**

(A, B) Representative blots of p-ERK and t-ERK in TG and WT primary neutrophils. (C, D) Detection and quantification of ROS by dihydroethidium (DHE) staining in dHL-60 cells transfected with si-S100A12 or pcDNA3.1-S100A12. (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. Scale bar, 10 μm. (G, H) The ROS inhibitor diphenyleineiodonium (DPI) reduced NET formation in neutrophils transfected with pcDNA3.1-S100A12. a: $P < 0.001$ vs pcDNA3.1-NC+0 μM DPI. b: $P < 0.001$ vs pcDNA3.1-S10012+0 μM DPI. c: $P < 0.001$ vs pcDNA3.1-S10012+1 μM DPI. (I–K) Representative blots of NOX1, NOX2, and NOX4 in dHL-60 cells transfected with si-S100A12 or pcDNA3.1-S100A12.

*Q5.3- Furthermore, it is important to inquire whether other immune cells, such as monocytes and macrophages, were also examined.*

**A5.3:** Thank you for the comment. As you pointed out, besides its high expression in the myocardial tissue of mice on Post-MI Day 1 after MI, a small amount of co-localization staining of S100A12 and the macrophage marker molecule CD68 was observed in the myocardial tissue of mice at Post-MI Day 7. This suggests that S100A12 may be involved in the regulation of macrophage inflammation (**Figure to reviewer 6**).

To clarify whether S100A12 in neutrophils affects post-MI cardiac function, we constructed a new mouse model with neutrophil-specific promoter overexpression of S100A12 (Item No. PO-GJS0220240100897-01, GemPharmatech LLC.) and detected impaired cardiac function in mice on Post-MI Day 14 due to time constraints in the construction of transgenic mice. Compared to myeloid overexpression, neutrophil overexpression significantly reduced mouse cardiac function, albeit without statistical difference. This suggests that the effect of S100A12 on post-MI cardiac function might mainly be attributable to its impact on neutrophils. These results have been included in **Figure to reviewer 7**.

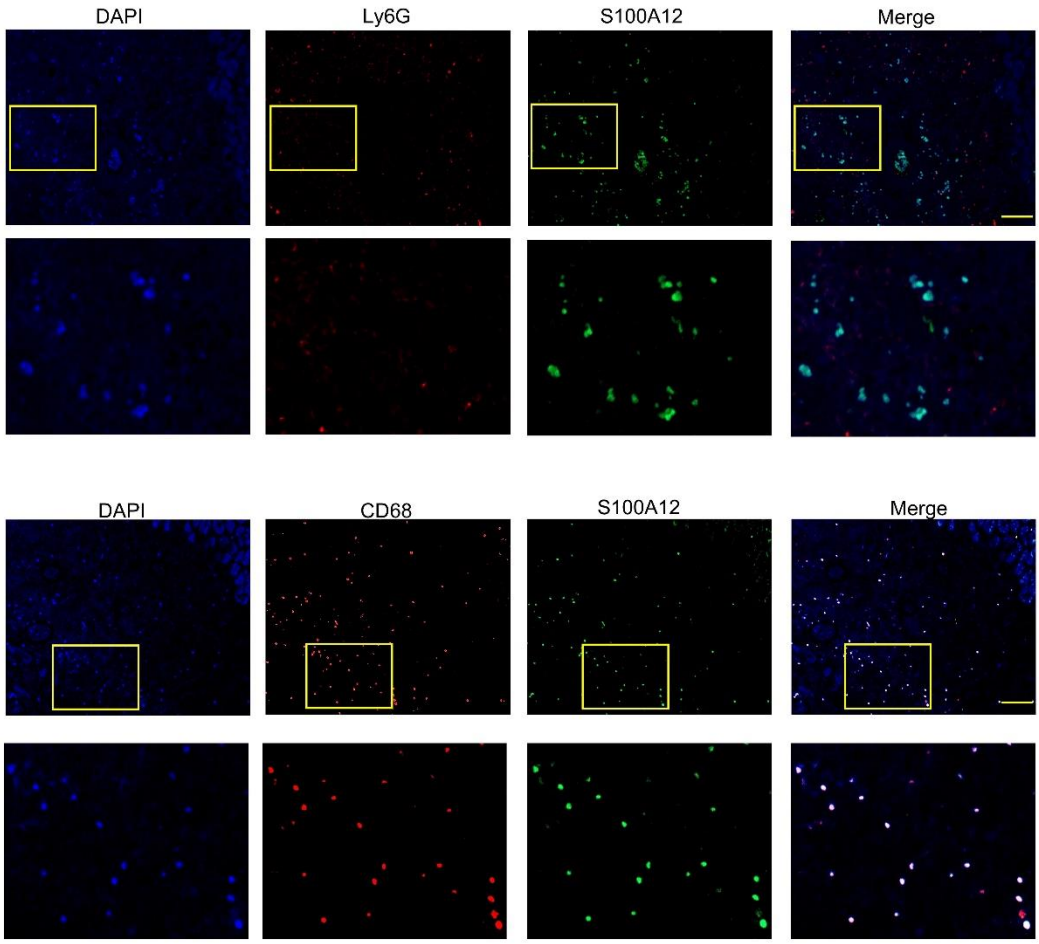



**Figure to reviewer 6. Overview images showing the presence of S100A12 in the infarct margin area on Post-MI Day 7.**

Immunofluorescence double staining of the neutrophil marker (Ly6G) / macrophage marker (CD68) and S100A12 in the infarct margin of TG and WT mice on Post-MI Day 7. Scale bar, 100 μm.

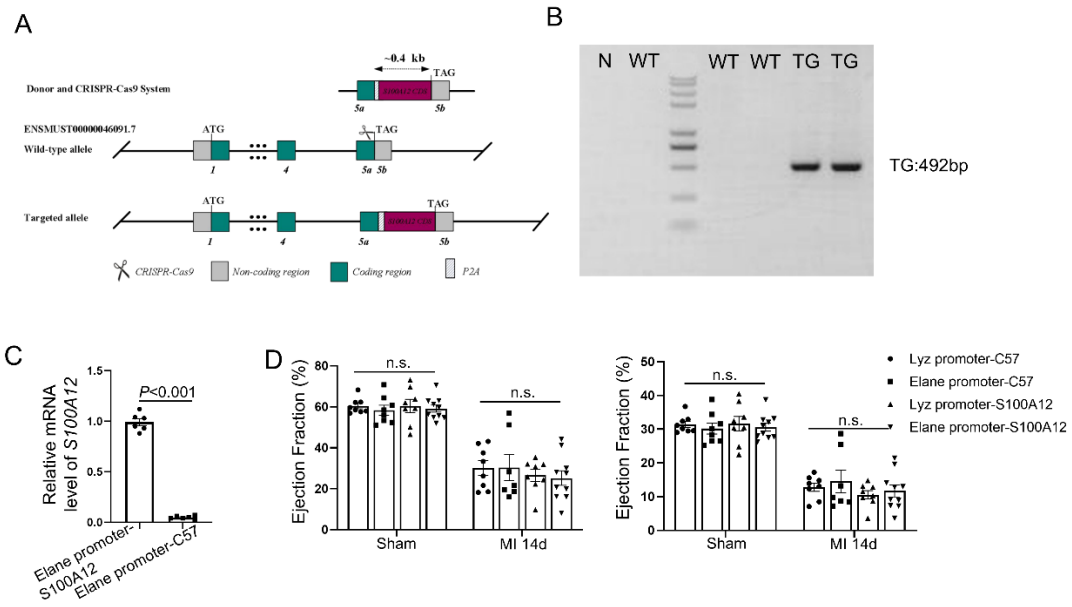



**Figure to reviewer 7. Generation of neutrophil-specific promoter overexpression of S100A12 in mice**.

(A) A schematic diagram of neutrophil-specific promoter (Elane promoter) S100A12 overexpression mice generation. (B) TG mice were identified using PCR analysis. (C) S100A12 mRNA expression level was analyzed in the bone marrow-derived neutrophils from TG and WT mice. (D) Echocardiographic parameters were compared between myeloid-specific promoted S100A12 gene knock-in mice and neutrophil-specific promoted S100A12 gene knock-in mice. Data are expressed as mean ± SEM.

*Q6-Did the authors check the fate of NETosed neutrophils in terms of apoptosis? As I initially suggested, it is important to test the kinetics of neutrophils/NETs at different time points post-MI for both NETs and apoptosis.*

**A6:** We thank the reviewer for this comment.

As shown in **Figure to reviewer 3M** and **3N**, the proportion of apoptotic cells was elevated in S100A12-overexpressing neutrophils, and inhibition of S100A12 downregulated the proportion of apoptotic cells. As S100A12 expression in neutrophils directly affects NET production, NETosed neutrophils are more likely to undergo apoptosis.

*Q7-Treatment of DNase injection was given onward Day7, but the lifecycle of neutrophils and NETs is not that long (Neutrophils infiltration, NETs formations and clearance happened within 48 day), we have observed this in lungs neutrophil peak start from 18 hours and peaked after 32 and last up to 72 hours. So how these time points have been determined.*

**A7:** We Thank you for your comment. We agree with the suggestion.

According to a literature review and our own experimental observations, the results confirmed that white blood cell infiltration peaked on Post-MI Day 1 and gradually subsided after 3 days. To better observe the inhibitory effect of DNase I on NETosis and reduce the side effects, we conducted a new interventional experiment. We performed the MI model again in WT and TG mice with DNase I injection for 3 days, as suggested by the reviewer. Echocardiographic analyses revealed that, compared to saline-injected mice, TG mice treated with DNase I showed improved ejection fraction (EF) and fractional shortening (FS) at the Post-MI Day 28, as shown in **Figure to reviewer 8A–8C.** Similarly, H&E and Masson staining showed that DNase I-injected mice demonstrated an obviously reduced infarct size and wall thickness compared with corn oil-injected TG mice; however, there was no significant difference between the WT groups (**Figure to reviewer 8D–8F**). The results have been added to **Supplementary Figure 3K–3Q** and to the Results section in the manuscript (page 10, line 8-13).

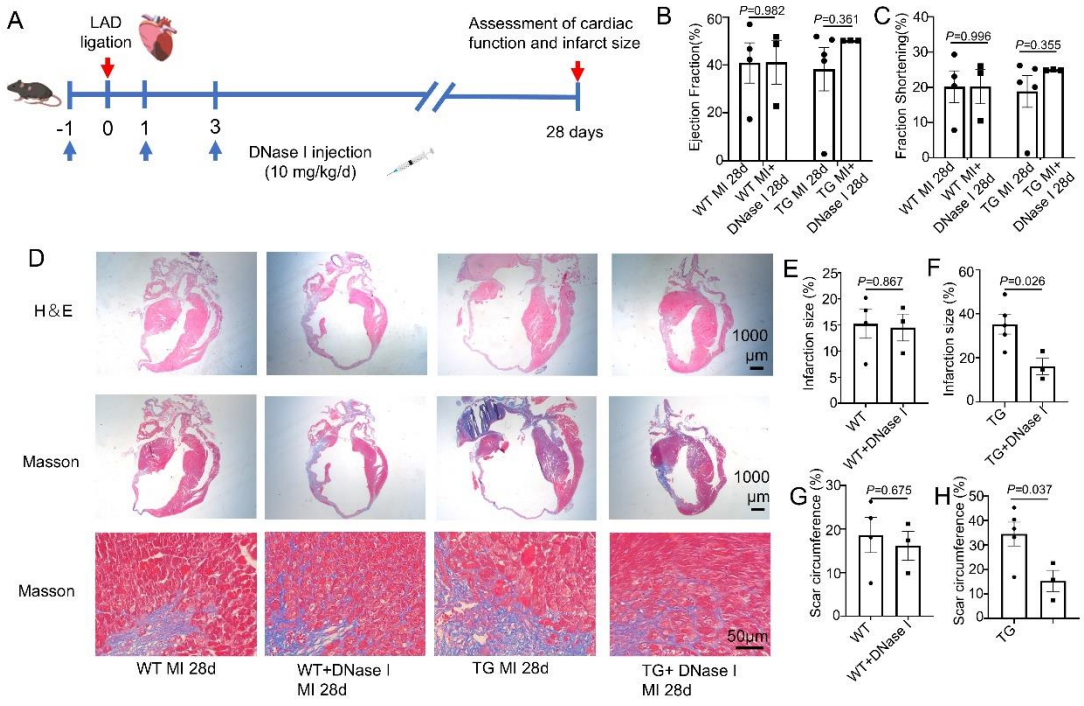



**Figure to reviewer 8. DNase I treatment improved post-MI cardiac function and ventricular remodeling in TG mice.**

(A) A schematic representation of the *in vivo* experiment. Mice were intraperitoneally injected with saline or DNase I (10 mg/kg/day) every other day from Day 1 before the MI operation to Post-MI Day 3. (B, C) Echocardiographic parameters were analysed in WT and TG mice treated with DNase I at Post-MI Day 28. (D) Representative Masson and H&E staining of cardiac tissues obtained from WT and TG mice at Post-MI Day 28 after intraperitoneal injection of DNase I. (E – H) Quantitative analysis of scar circumference and infarct size at Post-MI Day 28. Data are expressed as mean ± SEM.

*Q9- Its always better to have 60× zoomed image of NETs to really see the NETs and colocalization of NETs marker (MPO, CitH3 and NE).*

**A9:** We Thank you for your comment. As suggested by the reviewer, we have added 63× magnified photographs to replace **Figures 3A, 5J,** and **6I** and **Supplementary Figure 3A** in the manuscript, as shown in **Figure to reviewer 9**.

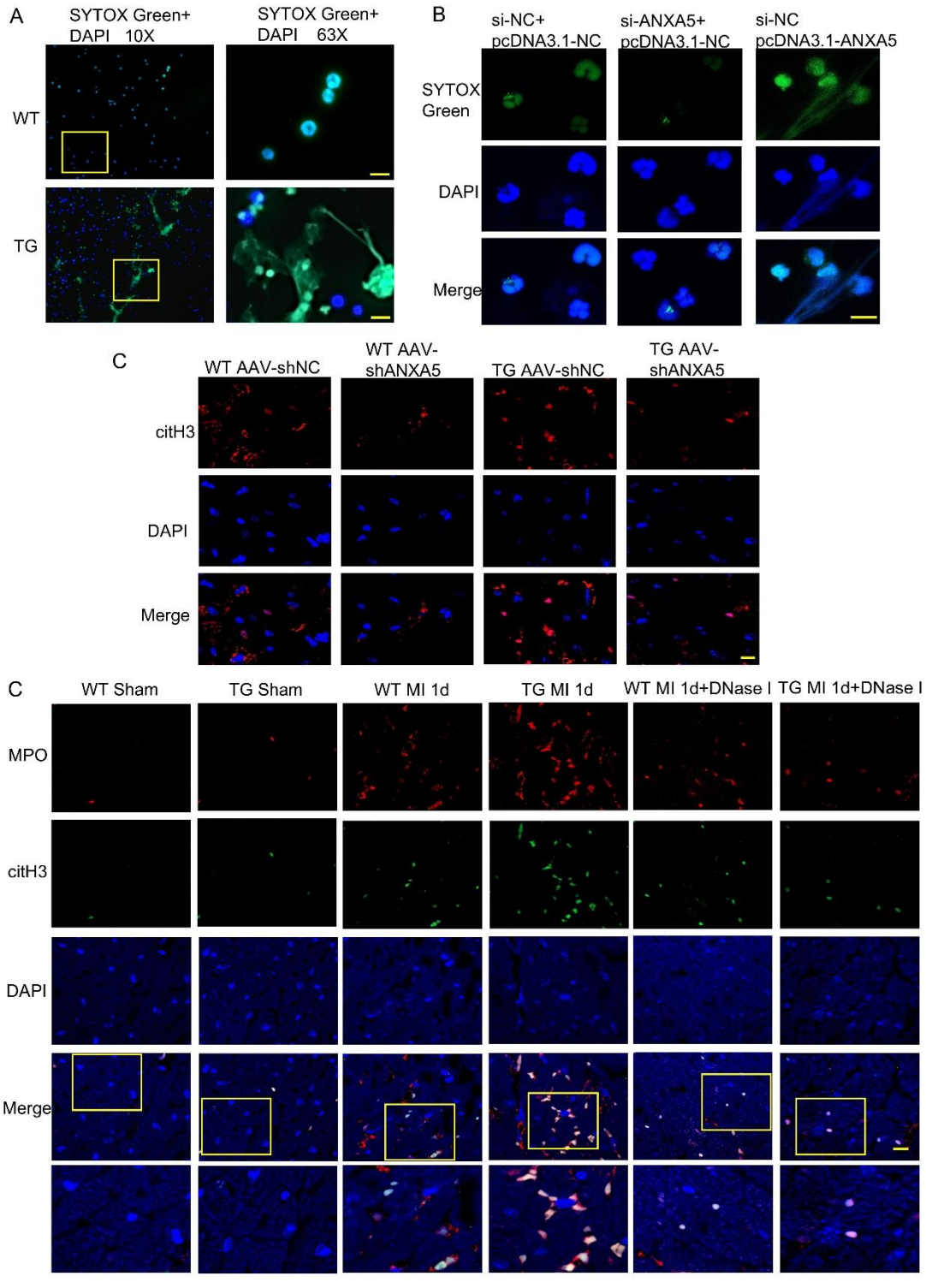


**Figure to reviewer 9. 63×-zoomed image to visualize NETs and colocalization of NET markers (MPO and citH3)**.

(A) SYTOX Green staining was performed to observe NET formation in BMDNs from TG and WT mice. (B) SYTOX Green staining was performed to observe NET formation in neutrophils with low or high ANXA5 expression. (C) Immunofluorescent staining of citH3 in WT and TG mice pretreated with AAV-shANXA5 or AAV-shNC on Post-MI Day 1. (D) Double immunofluorescence staining of MPO and citH3 in the infarct margin area of TG and WT mice treated with DNase I on Post-MI Day 1. Scale bar, 10 μm.

**RESPONSES TO THE COMMMENTS OF REVIEWER #2:**

***Q1***. *It is surprising that S100A12 is expressed in the mice until 4 weeks after myocardial infarction. Since the results that S100A12 can be found in neutrophils 1 day after myocardial infarction are convincing, which cells express S100A12 after 1week, when no neutrophils should be in the affected area? AN overview image showing the presence of S100A12 at the border will be useful.*

**A1:** Thank you for your comment. As suggested, we have added the following experiments: Based on the immunofluorescence staining results, we found that S100A12 was expressed in small amounts in the infarct border zone on Post-MI Day 7 and colocalized mainly with the monocyte marker CD68. The results are shown in **Figures to reviewer 6** and **7**.

***Q2***. *Can DNase I treatment reduce the infarction size and preserve the heart function after administrated to the TG mice? This is an important fact, since S100A12 can be found at later time points after myocardial infarction and can have secondary effects which can annulate the initial positive effect on inflammation (for example on fibrosis and remodeling).*

**A2:** Thank you for this comment.

In our original manuscript version, we reported that we used DNase I until Post-MI Day 7 because of its reported potential to increase neutrophil recruitment within 6 h and maintain elevated neutrophil counts (peaking at 24 h) throughout the 15 days following MI; the increase is especially significant in the first 7 days[7]. Although we detected significant inhibition of NETosis on Post-MI Day 1, we did not observe a significant improvement in cardiac function or ventricular remodeling on Post-MI Day 28 with 7-day DNase I injection.

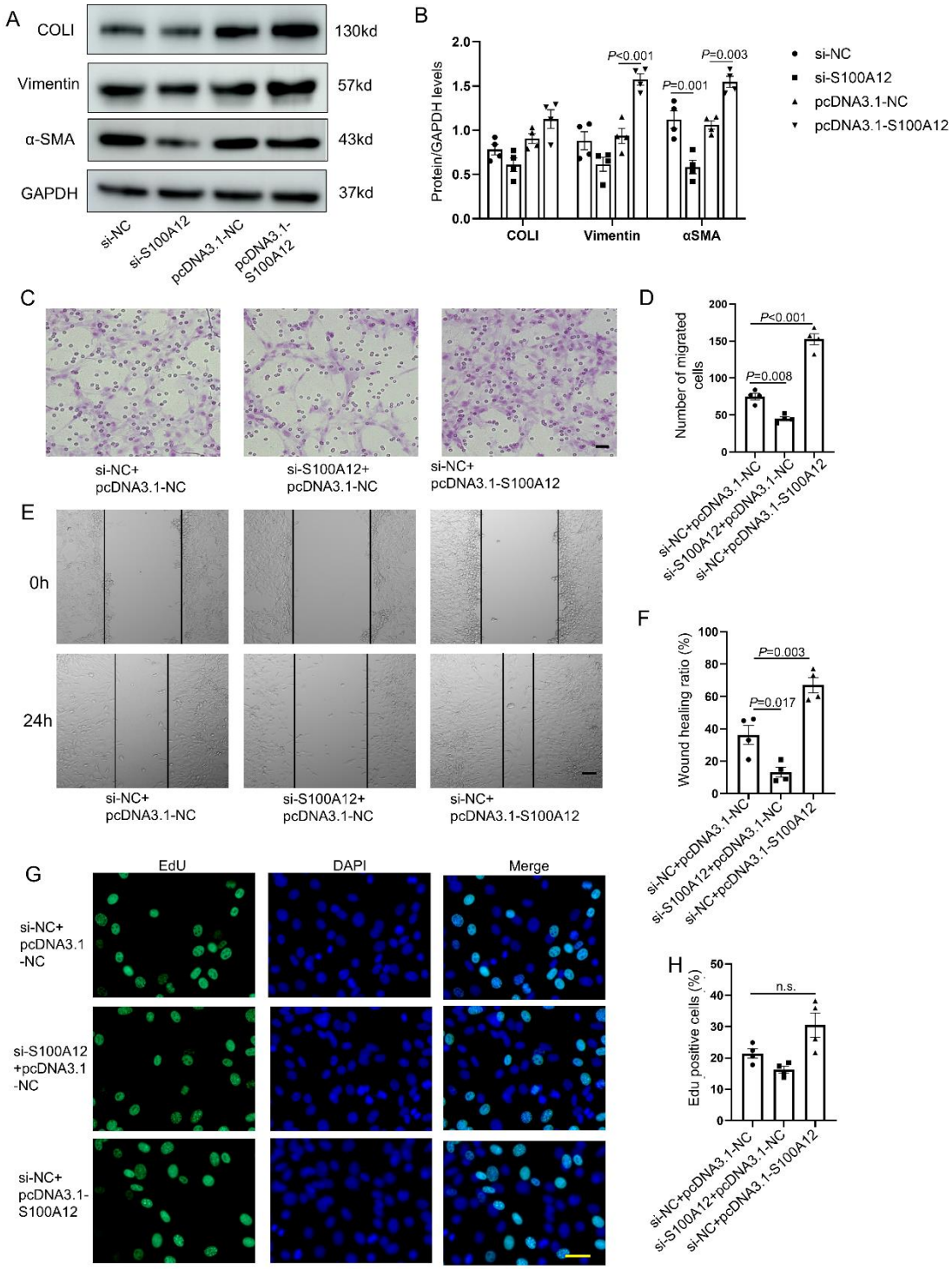
According to a literature review and our own experimental observations, the results confirmed that white blood cell infiltration peaked on Post-MI Day 1 and gradually subsided after 3 days. To better observe the inhibitory effect of DNase I on NETosis and reduce its side effects, we conducted a new interventional experiment. We re-performed a coronary artery ligation model in WT and TG mice with DNase I injection for 3 days, as suggested by Reviewer 1. Echocardiographic analyses revealed that TG mice treated with DNase I showed some improvement in the percentages of EF and FS at Post-MI Day 28, as compared to the saline-injected TG mice. H&E and Masson staining showed that DNase I-injected TG mice

demonstrated reduced infarct size and wall thickness compared to saline-injected mice; however, there was no significant difference between the WT groups. The results are shown in **Figure to reviewer 8** and **Supplemental Figure 3K–3Q**. This suggests that DNase I treatment can mitigate the damage to cardiac function that is induced mainly by S100A12-NETosis in the early stages after MI.

***Q3***. *Early after myocardial infarction, the proliferation and differentiation of fibroblasts is a crucial factor for proper healing and later scar formation. Since the presence S100A12 induce apoptosis in surrounding cells, what is the effect of the presence of S100A12 in neutrophils on proliferation and differentiation of fibroblasts?*

**A3:** Thank you for this comment.

To investigate whether the presence of S100A12 in neutrophils influenced the proliferation and differentiation of fibroblasts, we cocultured dHL-60 neutrophils with NIH 3T3 fibroblasts using polycarbonate membrane filters. As shown in **Figures to reviewer 10A** and **10B**, co-culture with S100A12-overexpressing neutrophils upregulated the protein levels of activated fibroblasts markers as COLI, Vimentin, and α-SMA. Moreover, fibroblasts co-cultured with neutrophils overexpressing S100A12 exhibited a higher migration capacity in wound healing and Transwell migration assays (**Figure to reviewer 10C–10F**). Cell proliferation was assessed by measuring incorporation of 5-ethynyl-2'-deoxyuridine (EdU) staining and propidium iodide (PI) staining using flow cytometry, following the manufacturer's instructions. However, we did not detect a significant effect of S100A12 expression in neutrophils on the proliferation of co-cultured fibroblasts (**Figure to reviewer 10G–10J**).


A
COLI
Vimentin
α-SMA
GAPDH
130kd
57kd
43kd
37kd
si-NC
si-S100A12
pcDNA3.1-NC
pcDNA3.1-S100A12
B
Protein/GAPDH levels
P<0.001
P=0.003
P=0.001
COLI
Vimentin
αSMA
si-NC
si-S100A12
pcDNA3.1-NC
pcDNA3.1-S100A12
C
si-NC+ pcDNA3.1-NC
si-S100A12+ pcDNA3.1-NC
si-NC+ pcDNA3.1-S100A12
D
Number of migrated cells
P<0.001
P=0.008
si-NC+pcDNA3.1-NC
si-S100A12+pcDNA3.1-NC
si-NC+pcDNA3.1-S100A12
E
0h
24h
si-NC+ pcDNA3.1-NC
si-S100A12+ pcDNA3.1-NC
si-NC+ pcDNA3.1-S100A12
F
Wound healing ratio (%)
P=0.003
P=0.017
si-NC+pcDNA3.1-NC
si-S100A12+pcDNA3.1-NC
si-NC+pcDNA3.1-S100A12
G
EdU
DAPI
Merge
si-NC+ pcDNA3.1 -NC
si-S100A12 +pcDNA3.1 -NC
si-NC+ pcDNA3.1-S100A12
H
Edu positive cells (%)
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si-S100A12+pcDNA3.1-NC
si-NC+pcDNA3.1-S100A12

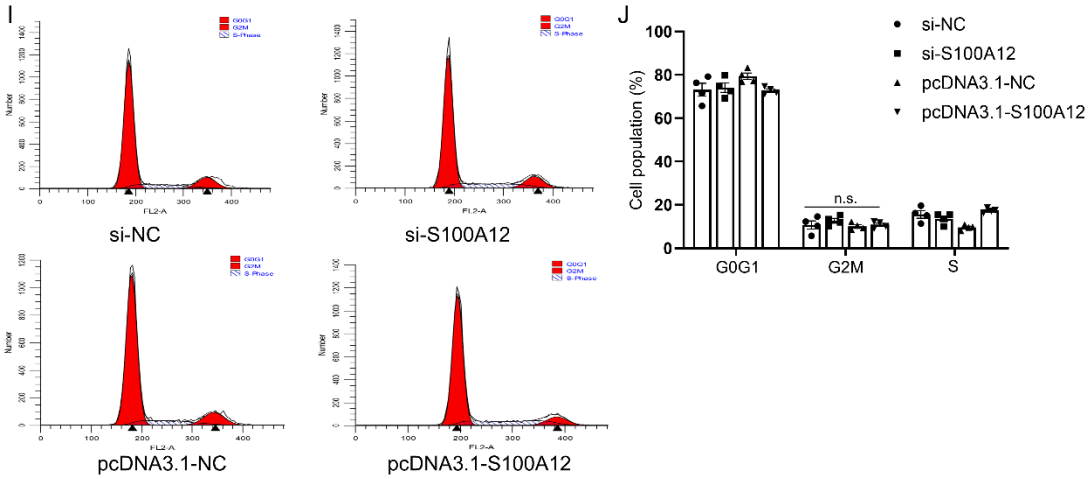



**Figure to reviewer 10. S100A12 expression in neutrophils affected the proliferation and differentiation of co-cultured fibroblasts.**

Cultured dHL-60 cells were transfected with si-S100A12 or pcDNA3.1-S100A12. (A–B) Western blot was conducted to detect the protein expression ratio of COLI, Vimentin, α-SMA and statistical results. (C–D) Transwell assay showing fibroblast migration ability in different groups. Scale bars, 50 μm. (E, F) Wound-healing experiments on fibroblasts co-cultured with neutrophils and statistical results. Scale bars, 20 μm. (G–H) Cell proliferation was measured by 5-ethynyl-2'-deoxyuridine (EdU) staining and quantification. Scale bars, 50 μm. (I, J) Cell-cycle analysis by PI staining and flow cytometry for co-cultured fibroblasts and quantitative measurement of the cell cycle phase. n=4.

***Q4**. The mechanism of S100A12/Annexin5 through calcium-channels and use of calcium-channel blockers is a very interesting and important finding, with direct clinical translation potential, despite the positive effects with the AAV-shANXA5. Can treatment with Verapamil in TG mice reverse the effect of S100A12 expression on cardiac function and infarction size?*

**A4:** Thank you for this comment.

As suggested by the reviewer, we performed MI surgery in WT and TG mice with verapamil injection on day 1 before MI and on days 1 and 3 after MI. From the survival curve analysis, we found that the injection of Verapamil significantly improved the mortality rate in the acute phase after MI and cardiac function on day 14 post MI in TG mice (**Figure to reviewer 11).**

Meanwhile, the expression of MPO and citH3 in verapamil-injected TG mice was significantly lower than that in corn oil-injected TG mice on Post-MI Day 1 (**Figure to reviewer 12** and **Supplemental Figure 7B–7G**). The protein levels of Bax and cleaved-caspase3 in verapamil-injected TG mice. The results of the ELISA showed significantly lower concentrations of NE, MPO, and the key enzyme PAD4 in the plasma of verapamil-injected

TG mice on Post-MI Day 1 compared with the vehicle group (**Figure to reviewer 12**). Western blotting and immunofluorescence staining revealed that the expression of NET components (citH3 and MPO) was higher in the vehicle group than in the verapamil-injected group of TG mice (**Figure to reviewer 12**).

We have added these results to **Figure 4K–4N** and **Supplemental Figure 7 (page 13, line 13–18)**. This suggests that the ability of verapamil, a calcium blocker, might be related to its ability to inhibit the production of S100A12-induced NETs. Taken together, these findings indicate that treatment with verapamil tends to improve post-MI cardiac function and ventricular remodeling. However, more data are needed to refine our conclusions.

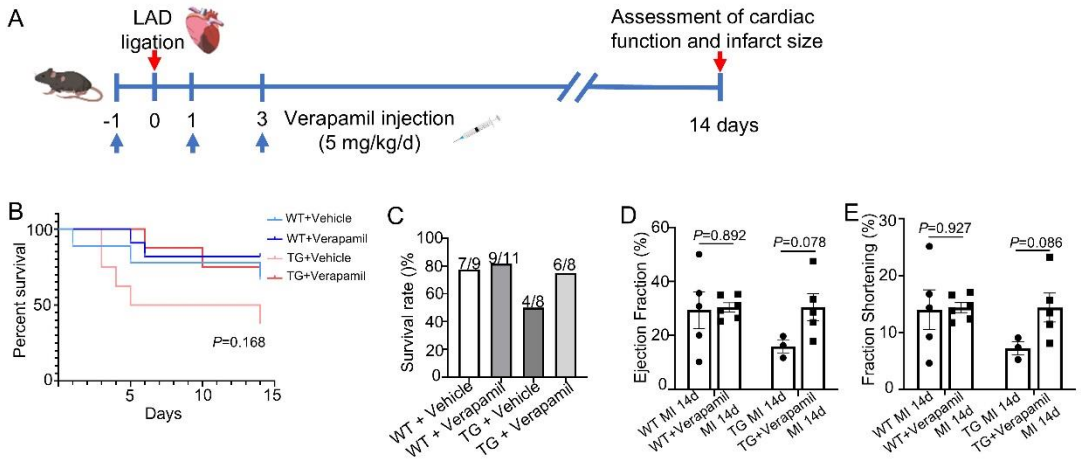
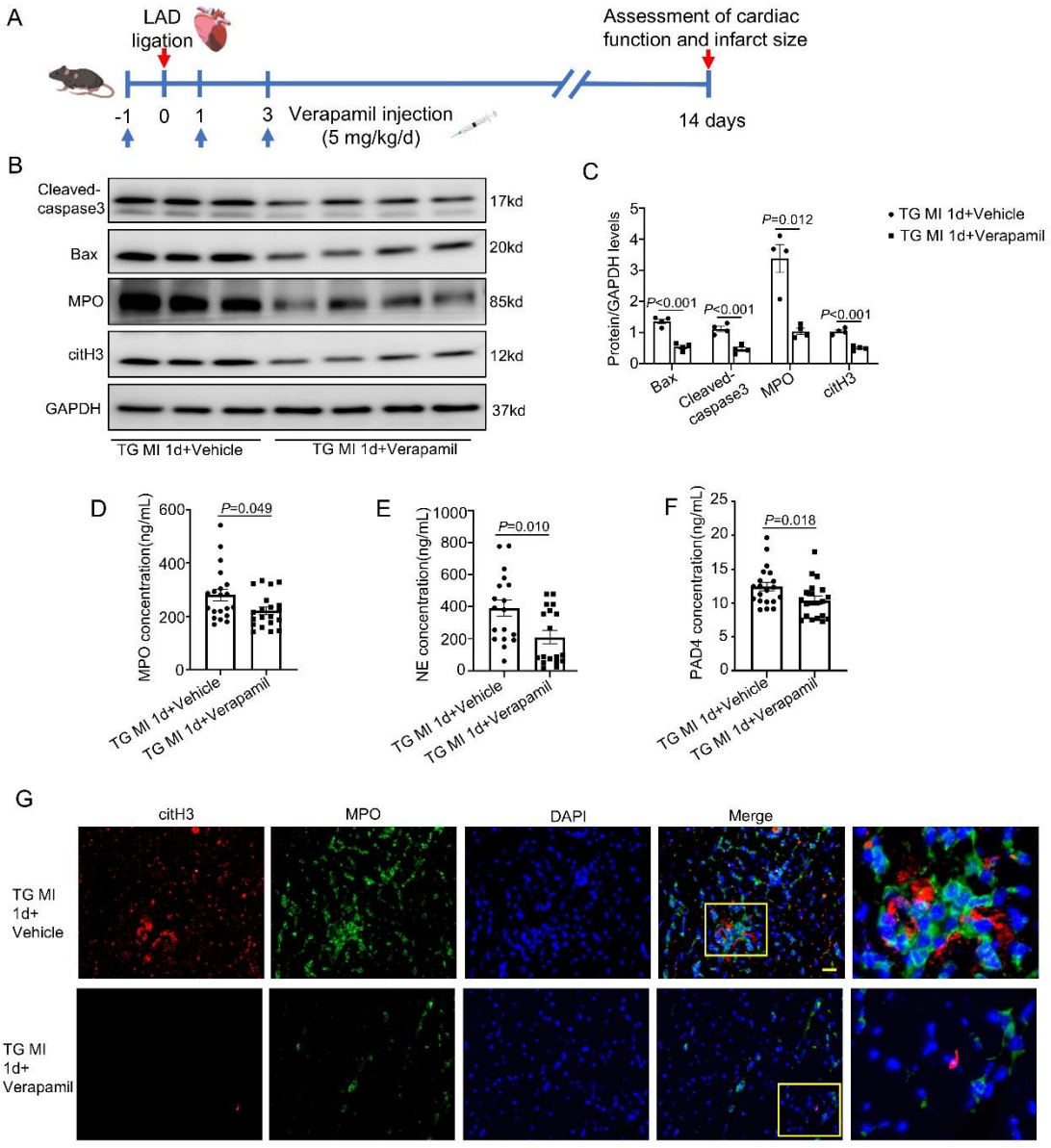



**Figure to reviewer 11. Treatment with verapamil improved the mortality rate and cardiac function at Post-MI Day 14.**

(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. (B, C) Survival curves and rates of WT and TG mice treated with verapamil for 28 days after MI. (D, E) Echocardiographic parameters were analysed in WT and TG mice treated with verapamil at Post-MI Day 28.




**Supplemental Figure 7-Figure to reviewer 12. Treatment with verapamil reduced neutrophil extracellular trap (NET) 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. (B, C) Representative blots of NETs- and apoptosis-related proteins and quantification in WT mice on Post-MI Day 1 with intraperitoneal injection of verapamil. (D–F) ELISA detection of MPO, NE, and PAD4 concentrations in the plasma of corn oil- or verapamil treated mice at 1 d post-MI. (G) Immunofluorescence double staining of MPO and citH3 in the infarct margin area of verapamil-treated mice on Post-MI Day 1. Data are expressed as mean ± SEM.

***Q5**. It is possible to follow if patients within lowest tertile have calcium-blockers in their medication?*

**A5:** We Thank you for your comment. As suggested by the reviewer, we followed the information of patients within the lowest tertile. We analysed the baseline conditions, medication details, and cardiac function values on the day of the AMI attack and found that the calcium blockers most patients took were nifedipine analogues. It is worth noting that compared to patients who did not take calcium blockers, patients who took calcium blockers regularly had lower plasma dsDNA concentrations and higher EF values on the day of the AMI attack, which might indicate relatively lower levels of NET production in the blood. However, these comparisons did not show statistically significant differences.

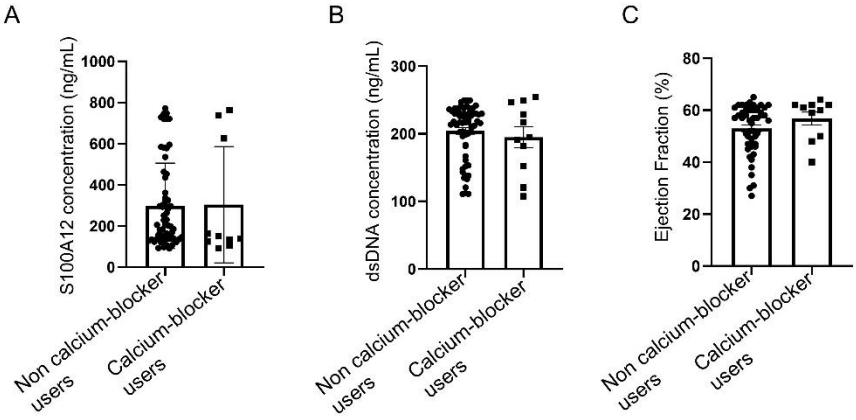



**Figure to reviewer 13. Plasma S100A12 and dsDNA concentrations and cardiac function analysis of patients within the lowest tertile.**

Comparison of S100A12 concentration (A), dsDNA concentration (B), and EF value (C) between calcium blocker and non-calcium blocker users in patients within the lowest tertile.

**Table to reviewer 1. Baseline characteristics of patients within the lowest tertile.**

| Variable | Non-calcium-blocker users (n=63) | Calcium-blocker users (n=11) | *P*-value |
|---|---|---|---|
| Sex, male | 50(79.3) | 8(72.7) | 0.69 |
| Age, years | 57.9±14.1 | 56.5±8.2 | 0.65 |
| Hypertension | 24(38.1) | 11(100) | <0.01 |
| Current smoking | 41(65.1) | 7(63.6) | 0.99 |
| Diabetes | 13(20.6) | 2(18.2) | 0.99 |
| Previous stroke | 7(11.1) | 2(18.2) | 0.61 |
| TG, mmol/dl | 1.9±1.0 | 1.7±0.4 | 0.27 |
| LDL-C, mmol/dl | 3.0±0.7 | 2.9±0.6 | 0.68 |
| HDL-C, mmol/dl | 1.0±0.2 | 0.9±0.1 | 0.03 |
| GLU, mmol/dl | 8.0±3.5 | 7.9±3.3 | 0.93 |
| WBC, $10^9$/L | 12.1±3.4 | 10.6±2.4 | 0.09 |
| hscTnT, ng/ml | 0.4±0.6 | 1.3±3.0 | 0.45 |
| Hs-CRP, mg/l | 15.4±32.3 | 5.5±4.3 | 0.11 |
| CK-MB, U/L | 84.6±118.7 | 58.0±71.3 | 0.32 |
| S100A12, ng/ml | 314.8±218.3 | 230.5±203.9 | 0.25 |
| dsDNA, ng/ml | 205.5±38.7 | 195.0±51.0 | 0.52 |

***Q6***. *The dual role of neutrophils in healing after myocardial infarction should be highlighted and comment in the manuscript.*

**A6:** Thank you for this comment.

It is well known that neutrophils can promote myocardial injury by releasing ROS, granular components, and pro-inflammatory mediators. Recent studies have shown that neutrophils form extracellular traps and produce extracellular vesicles (EVs) that increase inflammation and cardiac injury[8, 9]. Additionally, neutrophils enhance granulopoiesis, forming a positive feed-forward loop for neutrophil production and acute inflammation[10]. In contrast, emerging evidence has shown that neutrophils are indispensable for appropriate wound healing after MI. They promote inflammation resolution, angiogenesis[9], and scar formation by generating a wide array of pro-reparative factors, including neutrophil gelatinase-associated lipocalin, vascular endothelial growth factor A, cathelicidin, annexin A1, and specialized pro-resolving mediators[11, 12]. Neutrophils' recruitment has been shown to be an obligatory requirement for the successful switching from inflammatory response to its resolution. This dual function of neutrophils may explain unsuccessful attempts to translate anti-neutrophilic therapies into clinical settings.

There are no "good" or "bad" players in the realm of the immune cells involved in post-infarction myocardial remodeling. Each subset of immune cells present in the heart may either increase injury and inflammation or initiate healing of cardiac tissue. Maintaining a time-dependent balance in the function of immune cells, when inflammatory activity is followed

by timely switching on of the resolution and reparative mechanisms, is important for successful cardiac healing and remodeling following infarction.

According to your suggestion, we have added the following content to the Discussion (page 21, line 10-17):

It is well known that neutrophils can promote myocardial injury through ROS, granular components, and pro-inflammatory mediators. Recent studies have shown that neutrophils form extracellular traps that increase inflammation and cause cardiac injury. However, emerging evidence has revealed that neutrophils are indispensable for appropriate wound healing after MI, promoting inflammation resolution, angiogenesis, and scar formation. Maintaining a time-dependent balance in the work of neutrophils when inflammatory activity is followed by timely switching on the resolution and reparative mechanisms is important for successful post-MI cardiac healing and remodelling.

**Reference**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**REVIEWERS' COMMENTS**

Reviewer #1 (Remarks to the Author):

This is well laid study and authors have incorporated most of the suggestions. There are few suggestions to improve (optional) or can be discussed in discussion section.
1-Examining how S100A12 levels change over a more extended period could provide a better understanding of its dynamics and whether it has prolonged or delayed effects on NET formation and myocardial injury. Understanding/discussing these temporal patterns could clarify whether S100A12 plays a sustained role in post-MI inflammation or whether its effects are primarily early-stage.

2- Furthermore, a closer look into the specific mechanism of S100A12's interaction with Annexin A5 (ANXA5) to enhance calcium influx may reveal more detailed insights into the signaling pathway involved. Or exploring whether other proteins or signaling molecules play intermediary roles in this interaction might reveal potential targets for therapeutic intervention within the pathway.

3-Therapeutically, assessing pharmacologic inhibitors that target the S100A12 pathway or the S100A12-ANXA5 interaction could provide valuable translational insights. This can be done further by pathway and signaling analysis.

Reviewer #3 (Remarks to the Author):

Thank you for undressing all comments so carefully, I have no additional comments.
_____

**RESPONSES TO THE COMMENTS OF REVIEWER #1:**

***Q1***. *Examining how S100A12 levels change over a more extended period could provide a better understanding of its dynamics and whether it has prolonged or delayed effects on NET formation and myocardial injury. Understanding/discussing these temporal patterns could clarify whether S100A12 plays a sustained role in post-MI inflammation or whether its effects are primarily early-stage.*

**A1:** Thank you for your comment. According to your suggestions, we have added the following content to the Discussion (page 16, line 22-page 17, line 8):

*In present study, we found that S100A12 expression peaked within 1 d after MI and subsequently*

*declined, suggesting that the elevation of S100A12 is mainly concentrated in the early stage of MI injury. Moreover, we showed that S100A12 expression in neutrophils exacerbated cardiac functional impairment in mice after early MI, increasing apoptosis in heart tissue by elevating cytoplasmic and mitochondrial Ca2+concentration, which promoted suicidal NETosis formation. In addition to its role of initiating leukocyte dynamics and NETosis, other studies suggest that S100A12 may lead to an abnormal increase in MMPs activity by modulating $Zn^{2+1, 2}$and promoting atherogenesis.*

***Q2**. Furthermore, a closer look into the specific mechanism of S100A12's interaction with Annexin A5 (ANXA5) to enhance calcium influx may reveal more detailed insights into the signalling pathway involved. Or exploring whether other proteins or signalling molecules play intermediary roles in this interaction might reveal potential targets for therapeutic intervention within the pathway.*

**A2:** Thank you for your comment. According to your suggestions, we have added the following content to the Discussion (page 18, line 3-9):

*Future experimental studies will employ structural biology approaches, such as X-ray crystallography or cryo-electron microscopy, to map the binding interface between S100A12 and ANXA5. Additionally, functional assays using site-directed mutagenesis could clarify how this interaction regulates calcium signaling and NETs production. Elucidating these details will deepen our understanding of the signaling pathways involved and may uncover novel therapeutic targets.*

***Q3**. Therapeutically, assessing pharmacologic inhibitors that target the S100A12 pathway or the S100A12-ANXA5 interaction could provide valuable translational insights. This can be done further by pathway and signaling analysis.*

**A3:** Thank you for your comment. Based on your suggestion, we have added the following content to the Discussion (page 18, line 20- page 19, line 3):

*The S100A12-ANXA5-calcium axis shows significant potential for anti-inflammation and protection against the myocardial injury post MI. Evaluating pharmacologic inhibitors targeting the S100A12-ANXA5-calcium axis or the S100A12-ANXA5 interaction may provide valuable translational insights. Further studies are required to elucidate the regulatory mechanisms and signalling pathways involved in the S100A12-ANXA5 interaction.*

**References**

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