nature portfolio

Peer Review File

Spatiotemporal control of neutrophil fate to tune inflammation and repair for myocardial infarction therapy

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Kim and colleagues have designed an interesting study to determine the role of neutrophils in inflammation and cardiac repair following ischemic insults. Consideration of the comments will improve the current form of the manuscript, which contains multiple weaknesses as presented:

The introduction is extensive, covering multiple aspects of neutrophils. However, the key objective of nanoparticle, especially PLGA emphasis, needs to be highlighted in the presented manuscript.

The rationale for the CDK inhibitor (roscovitine) in the introduction is unclear given the role of neutrophils in the beginning of inflammation following ischemic insult.

The three-page introduction is a lengthy description of previous unrelated aspects. The key theme needs to be focused on with relevant outcomes.

NP characterization needs to consider the role of neutrophils in cardiac repair, considering their role in the reparative process. Please refer to PMID: 28158426.

The macrophage efferocytosis process is completed, but the presented markers are limited. Along with histological qualitative analysis, data using flow cytometry in the form of quantitative analyses is encouraged.

RC-NP directed immune modulation presented in histological markers. The number of total and various leukocyte subset analyses, including macrophage analyses, are encouraged using flow cytometry.

Functional parameters are providing limited information as presented. Thus, consider providing a smaller image strip with more cardiac strain details for improvement of function, if any.

Toxicity evaluations of NPs supported using histology have limited relevance. A specific and to-the-point description of histological outcomes is needed.

There is scope to improve the graphical sketch.

Figure 7B and C NO group figures are distorted likely during the file conversion, thus not evaluated.

Reviewer #2 (Remarks to the Author):

In this manuscript, Kim and colleagues developed poly(lactic-co-glycolic acid) nanoparticles loaded with Roscovitine and catalase with the aim of modulating the fate of neutrophils. Upon intravenous injection, these nanoparticles were internalized by circulating neutrophils that migrate to the infarcted heart. Subsequent activation by reactive oxygen species (ROS) triggered the release of Roscovitine, which

induced apoptosis in activated neutrophils and promoted macrophage polarization. As a result, this approach helps preserve cardiac function.
Overall, this study is well-structured and executed, yielding several encouraging findings. The design is straightforward. However, the mechanism of drug release requires further validation to ensure its robustness and reliability. In its current form, the work is not suitable for Nat Commun.

1, The central premise of the drug release mechanism is that “at the infarct area, H_2O_2 produced by activated neutrophils is converted to oxygen by catalase in RC NPs. The buildup of oxygen gas in RC NPs causes the nanoparticles to explode and rapidly release roscovitine…” However, it is important to note that the efficacy of Roscovitine release from RC NPs was evaluated using high concentrations of H_2O_2, up to 50 mM or 200 μM, in the Methods section. To justify the use of such high levels, it would be crucial to quantify the actual concentration of H_2O_2 produced by PMA-activated neutrophils (Fig. 3).
2, The author stated that “As RC NPs release roscovitine slowly in unactivated neutrophils and non-ROS conditions (Fig. 2E)”. The stability of RC NPs over a 7-day period is evident from Fig. 2C. Interestingly, within the first 12 hours, the RC NP+H_2O_2 group demonstrates a 60% release of Roscovitine, while other groups exhibit a release rate of over 30%, indicating a moderate but not slow release profile (Fig. 2E). This rapid initial release could be attributed to the degradation of PLGA. However, if this is not the case, it would be essential to explore alternative mechanisms for Roscovitine release, aside from the typical burst release associated with RC NPs.
3, As observed in Fig. 2G, RC NPs appear to be predominantly localized on the membrane of neutrophils.

RESPONSE TO REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Kim and colleagues have designed an interesting study to determine the role of neutrophils in inflammation and cardiac repair following ischemic insults. Consideration of the comments will improve the current form of the manuscript, which contains multiple weaknesses as presented:

1. The introduction is extensive, covering multiple aspects of neutrophils. However, the key objective of nanoparticle, especially PLGA emphasis, needs to be highlighted in the presented manuscript.

Response 1: We sincerely thank the reviewer for thoroughly reviewing our manuscript. We have now edited our introduction to focus more on the objective of PLGA nanoparticles (i.e., roscovitine delivery to activated neutrophils at the infarct site) as shown below.

[Introduction] (Page 4, Line 67): Here, we show that poly(lactic-co-glycolic acid) (PLGA) nanoparticles can be tuned to offer unique characteristics for neutrophil modulation. PLGA nanoparticles are readily taken up by neutrophils[1, 2, 3]. We modified PLGA nanoparticles to release drug in response to ROS produced under inflammatory conditions, which could allow for drug delivery to activated neutrophils at the infarct site. In this study, we fabricated hydrogen peroxide (H_2O_2)-responsive, roscovitine- and catalase-loaded PLGA nanoparticles (RC NPs). RC NPs can be readily taken up by neutrophils migrating to the infarcted heart. Activated neutrophils at the infarcted heart generate H_2O_2 which would be converted to oxygen by the catalase in RC NPs. The buildup of oxygen gas would cause internalized RC NPs to detonate and rapidly release roscovitine in the neutrophils (Fig. 1).

2. The rationale for the CDK inhibitor (roscovitine) in the introduction is unclear given the role of neutrophils in the beginning of inflammation following ischemic insult.

Response 2: We thank the reviewer for this comment. We have now clarified our rationale for the CDK inhibitor roscovitine in our introduction as shown below.

[Introduction] (Page 4, Line 76): Roscovitine is a cyclin-dependent kinase inhibitor that has been shown to effectively induce neutrophil apoptosis and has demonstrated to be safe in clinical trials[4, 5, 6]. By loading roscovitine inside RC NPs, activated neutrophils can undergo apoptosis specifically at the infarcted heart, thereby mitigating neutrophil-mediated cardiac damage. RC NP induced-apoptotic neutrophils can then promote cardiac macrophage efferocytosis and subsequent M2 polarization[7]. Previous strategies which employed small molecules or neutrophil-depleting antibodies to downregulate neutrophil activation, impair M2 polarization[8, 9, 10, 11]. In contrast, RC NPs could offer a more balanced mode of modulating neutrophils and macrophages, both of which are essential for cardiac repair.

3. The three-page introduction is a lengthy description of previous unrelated aspects. The key theme needs to be focused on with relevant outcomes.

Response 3:
We thank the reviewer for this suggestion. We agree that the introduction was too long, and contained unrelated aspects. We have now edited our manuscript to be more concise and to focus on the key theme with relevant outcomes. The word count of our introduction has been reduced by 25% (794 to 597 words).

Deleted (regarding neutrophils):
~~Inhibitors of NETosis, a form of neutrophil death involving the extrusion of NETs, have shown to reduce cardiac injury in preclinical MI models [12, 13]. Neutrophil stunning by the ß1-adrenergic-receptor antagonist metoprolol has shown to reduce infarct size in acute MI patients [14]. Neutrophil-based cardioprotective strategies that minimize cardiac injury are potentially advantageous over regenerative strategies such as stem cell-based therapies, microRNA (miRNA) targeting therapies, or fibroblast to cardiomyocyte reprogramming strategies that aim at repairing an infarcted heart that has undergone largely irreversible damage.~~

Shortened (regarding neutrophils):
Before: Upon arrival, neutrophils are activated by reactive oxygen species (ROS)-dependent pathways to secrete pro-inflammatory cytokines, chemokines, proteases, and neutrophil extracellular traps (NETs) [12, 15, 16]. Proteases such as neutrophil elastase (NE) and matrix metalloproteases (MMPs) directly incite cardiomyocyte death and cardiac tissue damage [17, 18]. Chemoattractants such as S100A8/A9, NETs, and cytokines such as tumor necrosis factor-α (TNF-α) amplify the inflammatory cascade through inflammatory monocyte recruitment and pro-inflammatory macrophage signaling [19, 20, 21]. Macrophages in turn exacerbate cardiac damage by secreting an array of pro-inflammatory cytokines including TNF-α and interleukins (IL)-6 and -12[22, 23].
After: (Page 3, Line 47): Upon arrival, neutrophils are activated by reactive oxygen species (ROS)-dependent pathways to secrete pro-inflammatory cytokines, chemokines, proteases, and neutrophil extracellular traps (NETs)[12, 15, 16], which incite cardiomyocyte death and tissue damage[17, 18] and amplify the inflammatory cascade through pro-inflammatory macrophage polarization[19, 20, 21].

Focus on key theme with relevant outcomes:
[Introduction] (Page 4, Line 85): We investigated the effect of RC NPs on initial cardiac pathology, cardiac remodeling, and heart function. Here, we demonstrate that a single intravenous injection of RC NPs in MI rats induces robust neutrophil apoptosis at the infarct heart. This leads to subsequent reparatory macrophage polarization and ultimately preserves heart function. This study highlights the therapeutic potential of targeting neutrophils to modulate inflammatory and reparatory processes following MI.

We believe the reviewer's comments regarding our introduction have helped to significantly improve our manuscript, and thank the reviewer once again for these suggestions.

4. NP characterization needs to consider the role of neutrophils in cardiac repair, considering their role in the reparative process. Please refer to PMID: 28158426.

Response 4: We thank the reviewer for this insightful suggestion. The study by Horckmans et al. (PMID: 28158426) demonstrates that indiscriminate depletion of neutrophils using antibodies worsens cardiac function because a reduction in cardiac neutrophils impairs M2 macrophage polarization and subsequent M2 macrophage-mediated cardiac repair. RC NPs are different from neutrophil-depleting antibodies as RC NPs do not indiscriminately deplete neutrophils. Instead, RC NPs induce timely apoptosis of activated neutrophils within the infarcted heart (Fig. 6a), which correlates with an increase in M2 cardiac macrophage polarization (Fig. 6c-e, Supplementary Fig. 9) and results in cardiac repair (Fig. 7b). This is because apoptotic neutrophils can provide a powerful stimulus for inducing reparatory M2 macrophages via efferocytosis[7].

RC NPs are able to promote neutrophil-mediated cardiac repair due to their unique characteristics. RC NP characterization data shows that RC NPs are readily taken up by circulating and cardiac neutrophils (Fig. 5a, b, f). After uptake, RC NPs induce apoptosis in cardiac neutrophils in infarcted hearts (Fig. 6a) as RC NPs are able to rapidly release roscovitine under H_2O_2 conditions (Fig. 2f). RC NP-induced apoptotic neutrophils can promote macrophage efferocytosis (Fig. 4a, b). Following efferocytosis, cardiac macrophages are polarized to the M2 phenotype (Fig. 6c-e, Supplementary Fig. 9). We have now edited the discussion section of our manuscript to better describe how the characteristics of RC NPs are advantageous to promoting neutrophil-mediated cardiac repair.

[Discussion] (Page 19, Line 392): RC NPs are capable of both downregulating overexuberant neutrophil signaling and promoting neutrophil-mediated repair. This can be ascribed to the unique characteristics of RC NPs. RC NPs are readily taken up by circulating and cardiac neutrophils (Fig 5a, b, f). After uptake, RC NPs induce apoptosis in cardiac neutrophils in infarcted hearts (Fig. 6a) as RC NPs are able to rapidly release roscovitine in H_2O_2 conditions (Fig. 2f). RC NP-induced apoptotic neutrophils can promote macrophage efferocytosis (Fig. 4a, b). Following efferocytosis, cardiac macrophages are polarized to the M2 phenotype (Fig. 6c-e, Supplementary Fig. 9).

5. The macrophage efferocytosis process is completed, but the presented markers are limited. Along with histological qualitative analysis, data using flow cytometry in the form of quantitative analyses is encouraged.

Response 5: We thank the reviewer for this suggestion. We have now quantitatively analyzed cardiac macrophages 3 days and 5 days post-MI using flow cytometry and found a significant reduction in M1 markers CD86 and CD80 in cardiac macrophages in RC NP-treated animals. Our revised data are shown below.

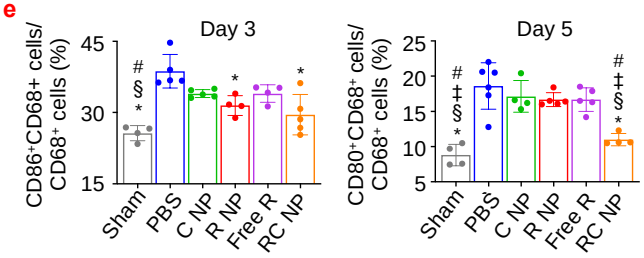
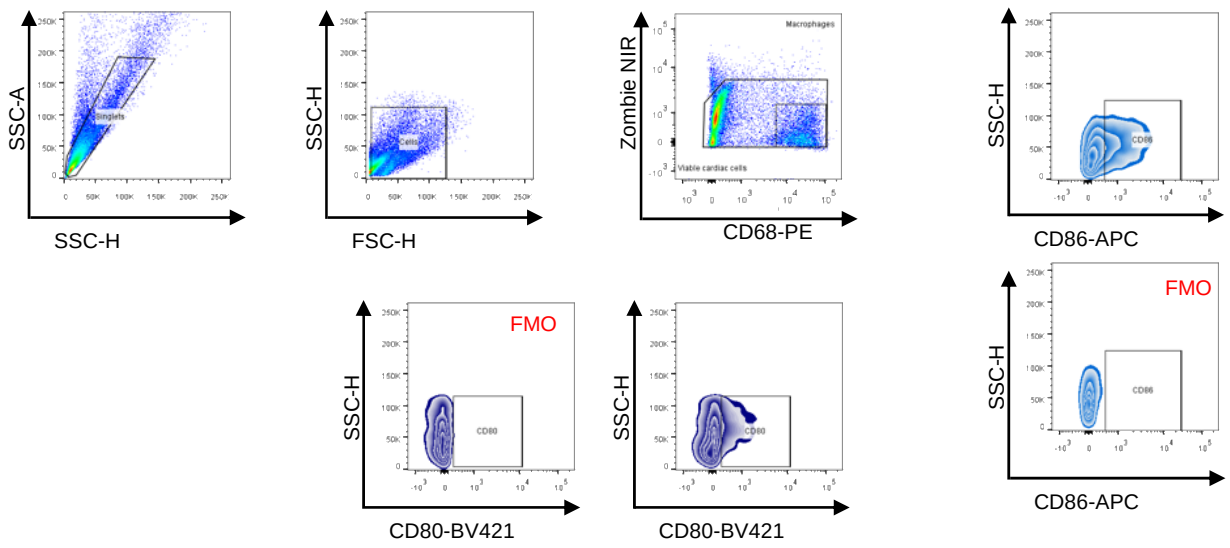


Fig. 6e CD86 and CD80 expression in macrophages ($CD68^+$ cells) 3 and 5 days after MI as determined by flow cytometry (n = 4-6). *p < 0.05 vs PBS; §p < 0.05 vs C NP; ‡p < 0.05 vs R NP; #p < 0.05

vs Free R; ⊤p < 0.05 vs RC NP; ⊥p < 0.05 vs Sham. All data presented as mean ± SD. One-way ANOVA was used for all comparisons.



Supplementary Fig. 10. Analysis of macrophage phenotypes in the hearts of MI rats. Representative scatter plots for Fig. 6e. Macrophages were defined as CD68+ cells.

[Results] (Page 13, Line 252): Flow cytometric analysis of cardiac macrophages also showed that RC NP treatment led to a significant decrease in expression of M1 markers CD80 and CD86 in cardiac macrophages ($CD68^+$ cells) (Fig. 6e, Supplementary Fig. 10).

[Methods] (Page 33, Line 692):

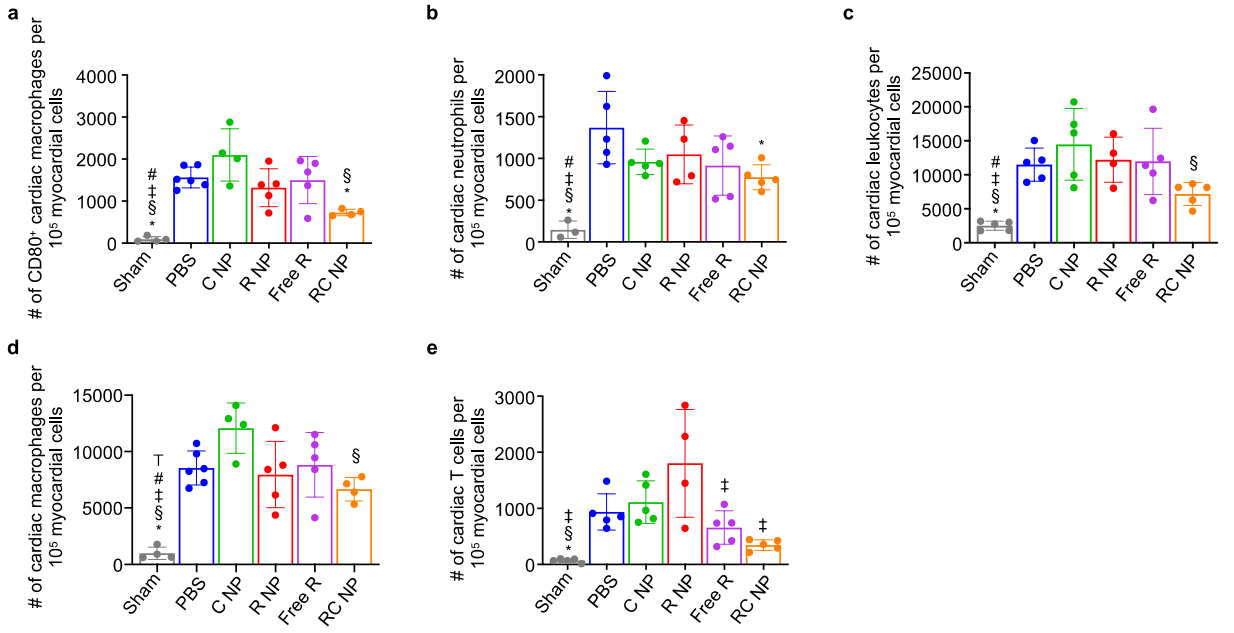
Cardiac leukocyte analysis

Hearts were dissected with perfusion, minced, and digested with 2 mg/ml of Collagenase Type IV and 1.2 U/ml of Dispase II in RPMI medium. Heart tissues were homogenized using a gentleMACS™ Dissociator and filtered through a 100 μm cell strainer. Samples were incubated with 100 μg/ml of anti-mouse IgG (Invitrogen) for 15 minutes at 4°C to prevent nonspecific binding. Cell suspensions were then stained with Zombie NIR (Biolegend) for 15 minutes at room temperature. Afterwards, cell suspensions were incubated at 4°C with fluorophore-conjugated antibodies. Anti-rat CD45, CD68, CD3, and CD86 antibodies were purchased from Biolegend, anti-rat His48 antibody was purchased from Invitrogen, and anti-rat CD80 antibody was purchased from BD Biosciences. Tru-Nuclear™ Transcription Factor Buffer Set (Biolegend) was used according to the manufacturer's instructions when permeabilization was required.

6. RC-NP directed immune modulation presented in histological markers. The number of total and various leukocyte subset analyses, including macrophage analyses, are encouraged using flow cytometry.

Response 6: We thank the reviewer for this suggestion. To gain insight into the various leukocyte subsets in the myocardium after RC NP treatment in MI rats, we analyzed leukocytes via flow cytometry 5 days after MI. RC NP treatment resulted in a significant decrease in the numbers of M1 ($CD80^+$) macrophages and neutrophils per 10^5 myocardial cells compared with the PBS control group.

However, we found that RC NP treatment did not affect the numbers of total leukocytes ($CD45^+$ cells), macrophages, and T cells per 10^5 myocardial cells compared with the PBS control group. These results further reinforce our hypothesis that RC NPs exert their therapeutic effects by modulating cardiac neutrophils and the phenotype of cardiac macrophages. Our results are shown below.



Supplementary Fig. 11. Number of various leukocyte subsets per 10^5 myocardial cells in the hearts of MI rats. Numbers of **a** M1 ($C80^+$) cardiac macrophages, **b** cardiac neutrophils, **c** cardiac leukocytes, **d** cardiac macrophages, and **e** cardiac T cells per 10^5 myocardial cells 5 days after MI as determined by flow cytometry (n =4-6). *p < 0.05 vs PBS; §p < 0.05 vs C NP; ‡p < 0.05 vs R NP; #p < 0.05 vs Free R; ⊤p < 0.05 vs RC NP. All data presented as mean ± SD. One-way ANOVA was used for all comparisons.

[Results] (Page 13, Line 254): Five days after MI, the numbers of M1 (CD80+) macrophages and neutrophils were significantly decreased in the hearts of RC NP-treated rats compared with that of PBS-treated rats (Supplementary Fig. 11a, b), while the numbers of total leukocytes, macrophages, and T cells in the hearts of MI rats were unchanged by RC NP treatment (Supplementary Fig. 11c-e). These data further reinforce our hypothesis that RC NPs exert their therapeutic effects by modulating cardiac neutrophils and the phenotype of cardiac macrophages.

[Methods] (Page 33, Line 692):

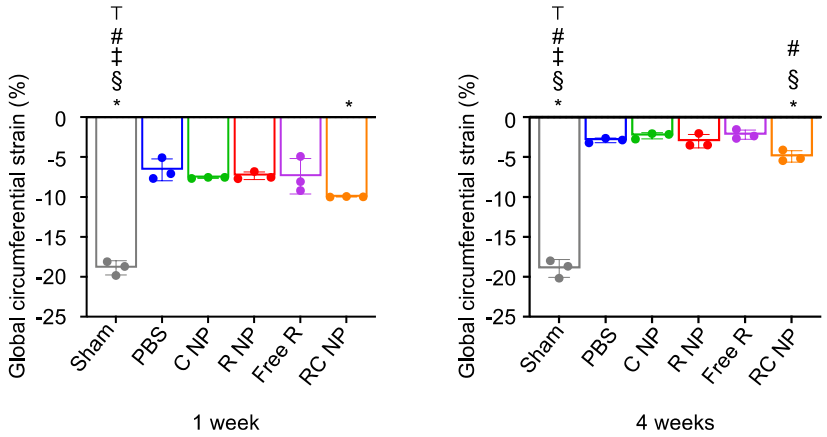
Cardiac leukocyte analysis

Hearts were dissected with perfusion, minced, and digested with 2 mg/ml of Collagenase Type IV and 1.2 U/ml of Dispase II in RPMI medium. Heart tissues were homogenized using a gentleMACS™ Dissociator and filtered through a 100 μm cell strainer. Samples were incubated with 100 μg/ml of anti-mouse IgG (Invitrogen) for 15 minutes at 4°C to prevent nonspecific binding. Cell suspensions were then stained with Zombie NIR (Biolegend) for 15 minutes at room temperature. Afterwards, cell suspensions were incubated at 4°C with fluorophore-conjugated antibodies. Anti-rat CD45, CD68, CD3, and CD86 antibodies were purchased from

Biolegend, anti-rat His48 antibody was purchased from Invitrogen, and anti-rat CD80 antibody was purchased from BD Biosciences. Tru-Nuclear™ Transcription Factor Buffer Set (Biolegend) was used according to the manufacturer's instructions when permeabilization was required.

7. Functional parameters are providing limited information as presented. Thus, consider providing a smaller image strip with more cardiac strain details for improvement of function, if any.

Response 7: We thank the reviewer for this suggestion. In our revised manuscript, we have now measured global circumferential strain, an index of left ventricular (LV) function, in MI rats 1 and 4 weeks after MI. In our revised data, we found significant preservation of global circumferential strain in RC NP-treated MI rats both 1 and 4 weeks after MI compared with PBS-treated MI rats. The results of our experiments are shown below.



Supplementary Fig. 13. Cardiac strain analysis 4 weeks after MI. Global circumferential strain measurements 1 and 4 weeks after MI (n = 3). *p < 0.05 vs PBS; §p < 0.05 vs C NP; ‡p < 0.05 vs R NP; #p < 0.05 vs Free R; ⊤p <0.05 vs RC NP. All data presented as mean ± SD. One-way ANOVA was used for comparisons.

[Results] (Page 14, Line 288): In addition, RC NP-treated MI rats showed a significant preservation of global circumferential strain, an index of LV function, 1 and 4 weeks post-MI compared with PBS-treated MI rats (Supplementary Fig. 13).

[Methods] (Page 30, Line 640):

Cardiac strain analysis

Global circumferential strain was derived parasternal short-axis views, recorded using a transthoracic echocardiography system equipped with an 11.0 MHz 12S-RS Sector Probe (Vivid™ iq, GE Healthcare, Chicago, IL, USA). Sampling points were manually placed along the epicardial and endocardial layers during the end-systolic period. Using GE EchoPAC v204 software, tissue speckles were identified and tracked throughout the cardiac cycle on a frame-by-frame basis, allowing the calculation of the global circumferential strain.

8. Toxicity evaluations of NPs supported using histology have limited relevance. A specific and to the-point description of histological outcomes is needed.

Response 8: We thank the reviewer for drawing our attention to this matter. To provide a more to the point description of histological outcomes, we have revised our manuscript as shown below.

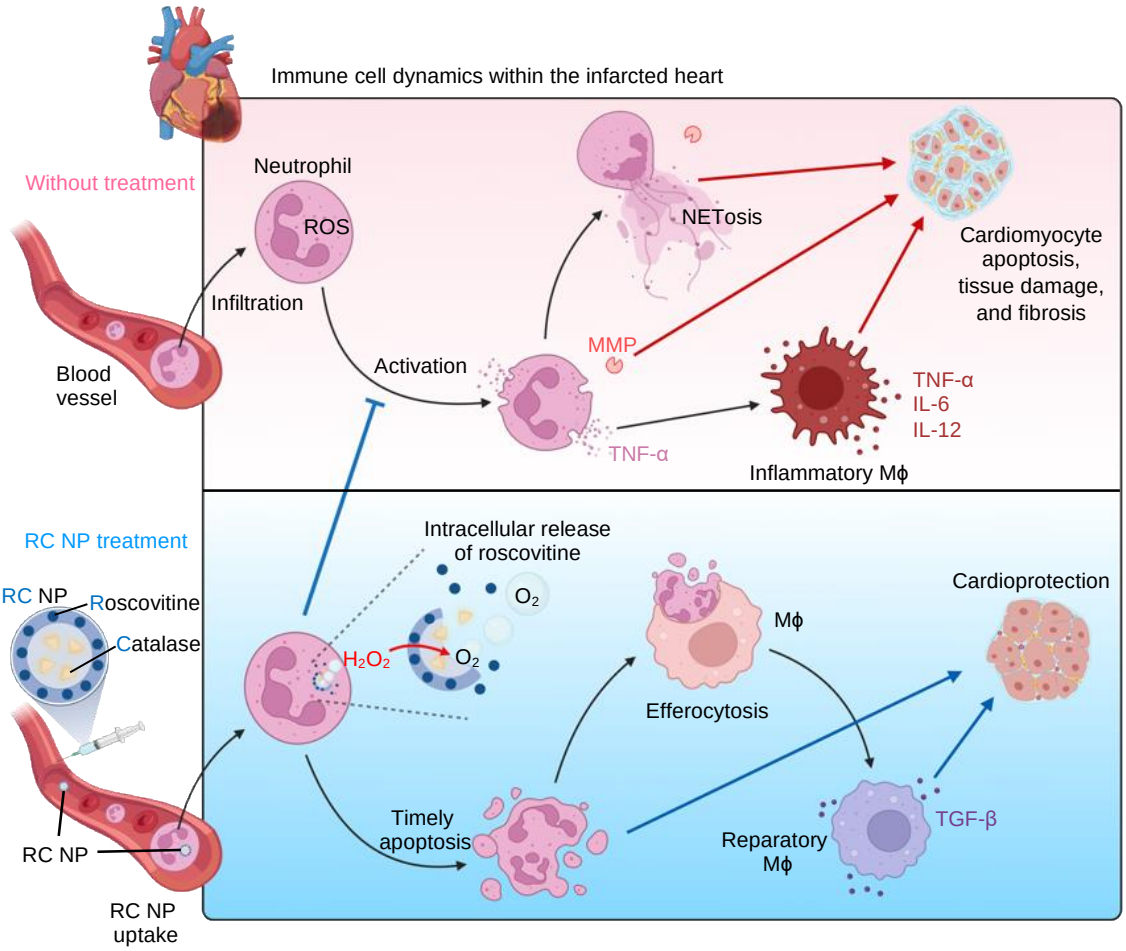
Before: Histological analysis of major organs also showed no noticeable differences between non-treated and RC NP-treated rats (Fig. 10E).

After: (Page 17, Line 343) Histological analysis showed that RC NPs did not induce discernable toxicity in major organs (Fig. 10e).

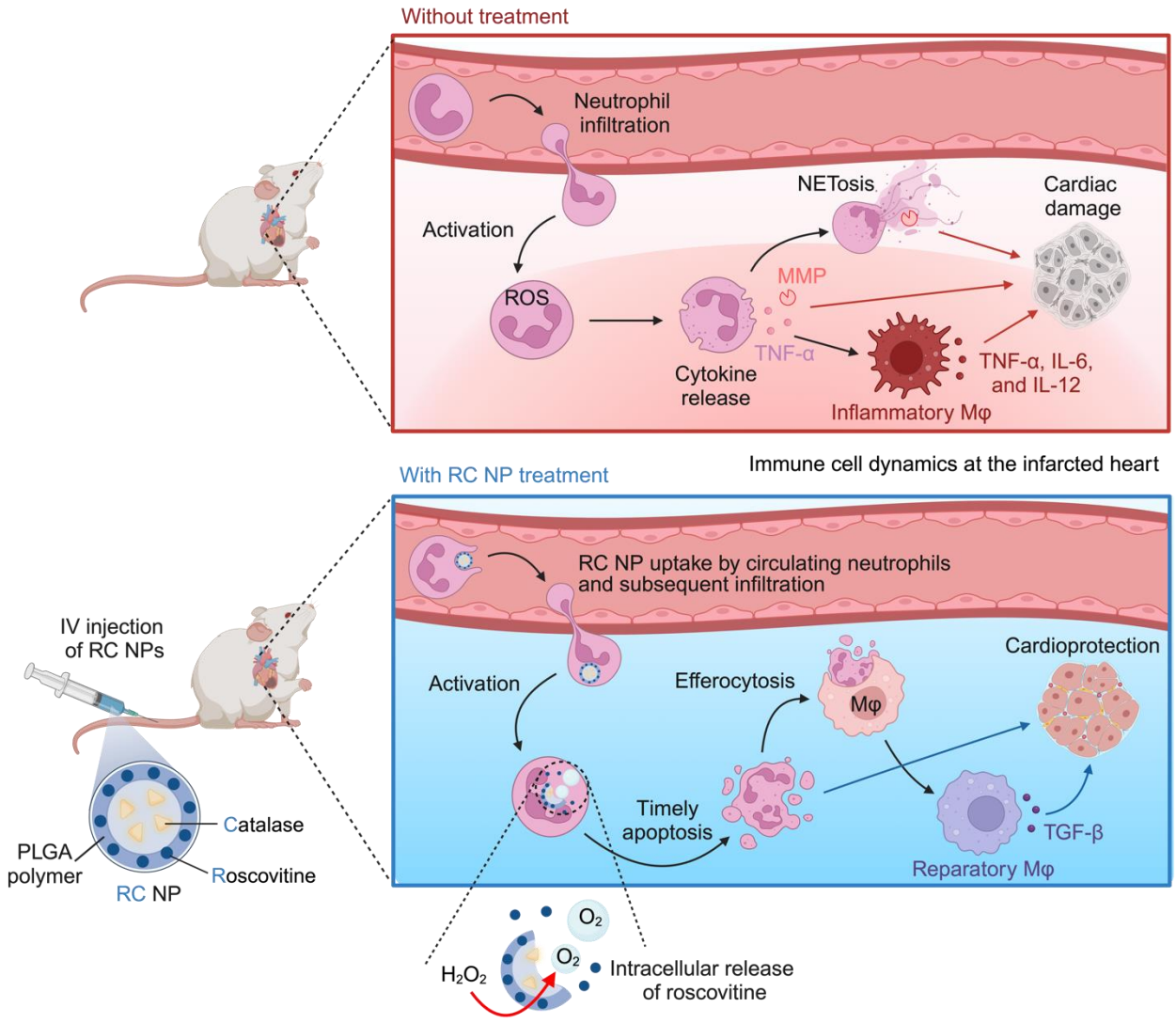
9. There is scope to improve the graphical sketch.

Response 9: We thank the reviewer for the suggestion. We have updated our graphical sketch in Fig. 1 as shown below. Specifically, we scaled up our sketch to better fit figure dimensions and simplified several aspects to improve contrast between immune cell dynamics with and without RC NP treatment.

Before:

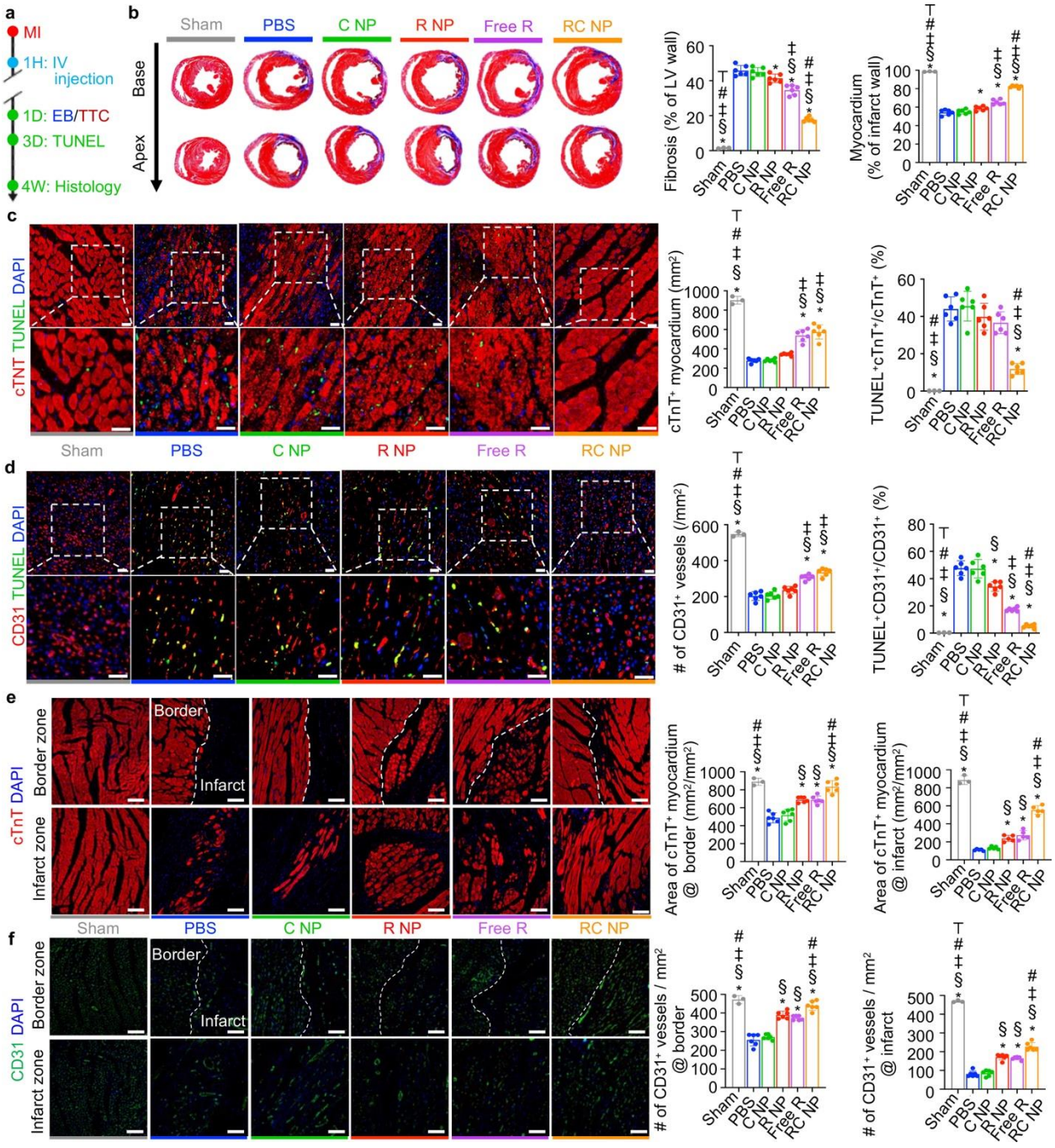


After:



10. Figure 7B and C NO group figures are distorted likely during the file conversion, thus not evaluated.

Response 10: We thank the reviewer for drawing our attention to this matter. It appears that there was image distortion during file conversion. We have added these images below for the reviewer to properly evaluate.


a
MI
1H: IV injection
1D: EB/TTC
3D: TUNEL
4W: Histology
b
Sham
PBS
C NP
R NP
Free R
RC NP
Base
Apex
Fibrosis (% of LV wall)
Myocardium (% of infarct wall)
c
cTNT TUNEL DAPI
cTnT⁺ myocardium (mm²)
TUNEL⁺cTnT⁺/cTnT⁺ (%)
d
CD31 TUNEL DAPI
# of CD31⁺ vessels (/mm²)
TUNEL⁺CD31⁺/CD31⁺ (%)
e
cTnT DAPI
Border zone
Infarct zone
Border
Infarct
Area of cTnT⁺ myocardium @ border (mm²/mm²)
Area of cTnT⁺ myocardium @ infarct (mm²/mm²)
f
CD31 DAPI
# of CD31⁺ vessels / mm² @ border
# of CD31⁺ vessels / mm² @ infarct

Reviewer #2 (Remarks to the Author):

In this manuscript, Kim and colleagues developed poly(lactic-co-glycolic acid) nanoparticles loaded with Roscovitine and catalase with the aim of modulating the fate of neutrophils. Upon intravenous injection, these nanoparticles were internalized by circulating neutrophils that migrate to the infarcted heart. Subsequent activation by reactive oxygen species (ROS) triggered the release of Roscovitine, which induced apoptosis in activated neutrophils and promoted macrophage polarization. As a result, this approach helps preserve cardiac function. Overall, this study is well-structured and executed, yielding several encouraging findings. The design is straightforward. However, the mechanism of drug release requires further validation to ensure its robustness and reliability. In its current form, the work is not suitable for Nat Commun.

1, The central premise of the drug release mechanism is that "at the infarct area, H2O2 produced by activated neutrophils is converted to oxygen by catalase in RC NPs. The buildup of oxygen gas in RC NPs causes the nanoparticles to explode and rapidly release roscovitine…" However, it is important to note that the efficacy of Roscovitine release from RC NPs was evaluated using high concentrations of H2O2, up to 50 mM or 200 μM, in the Methods section. To justify the use of such high levels, it would be crucial to quantify the actual concentration of H2O2 produced by PMA-activated neutrophils (Fig. 3).

Response 1: We sincerely thank the reviewer for this insightful suggestion. As our nanoparticles act inside neutrophils, intracellular H_2O_2 concentrations would be relevant to our study. However, due to the limitations of current methods to detect intracellular concentrations of ROS and H_2O_2, intracellular H_2O_2 concentrations cannot be determined in activated neutrophils. Assays that use small molecule substrates that react with H_2O_2 in the presence of horseradish peroxidase (HRP) to produce a fluorophore (such as the Amplex™ Red Assay) cannot be used to measure intracellular H_2O_2 concentrations as (1) all the small molecule fluorophore precursors are membrane impermeable[24 25] and (2) H_2O_2 cannot be readily extracted from cell lysates because of its instability (per manufacturer's instructions, Thermo Fisher, #A22188). The H2DCFDA assay, which is commonly used to detect intracellular ROS qualitatively, cannot be used to quantify ROS levels. Also, this assay detects several types of ROS in addition to H_2O_2.

A recent study demonstrated that PMA-activated neutrophils produce extracellular H_2O_2 concentrations of roughly 65 μM[26]. Also, an in vivo study involving forebrain ischemia reperfusion injury in rats (a model similar to ours) showed that extracellular H_2O_2 concentration at the injury site was roughly 100 μM[27]. Our nanoparticles operate inside neutrophils, and intracellular H_2O_2 concentration is expected to be higher than extracellular H_2O_2 concentration (>65 μM). Therefore, we have reperformed our release profile experiment in Fig. 2f at 100 μM H_2O_2.

We used 50 mM H_2O_2 (a relatively high concentration) in Fig. 2e (the experiment to evaluate oxygen production by RC NPs due to RC NP reaction with H_2O_2) because the oxygen probe used to detect oxygen generation in Fig. 2e has poor sensitivity (it has a range of error of ± 0.225 ppm). This probe cannot detect small changes in oxygen concentration. Therefore, we did not apply low concentrations of H_2O_2 for this experiment (Fig. 2e). Instead, to show that our nanoparticles can react with H_2O_2 at low concentrations, we have now performed an additional experiment measuring H_2O_2 concentrations after reaction with nanoparticles (Fig. 2d). The data indicate that our nanoparticles can readily react with low (5-80 μM) concentrations of H_2O_2. Our data are shown below.

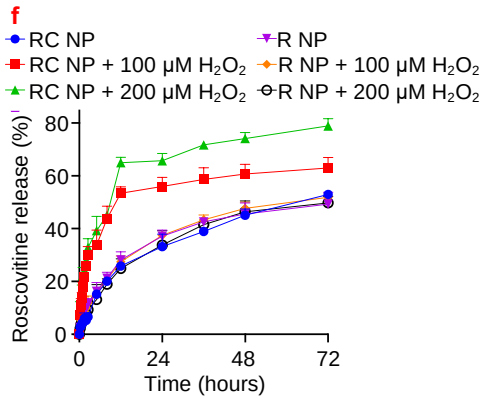


Fig. 2f Accelerated roscovitine release from RC NPs under H_2O_2 conditions (n = 3).

[Results] (Page 6, Line 102): Extracellular H_2O_2 concentration of phorbol-12-myristate-13-acetate (PMA)-activated neutrophils is roughly 65 μM[26]. Also, extracellular H_2O_2 concentration can reach up to 100 μM after ischemia reperfusion injury[27]. Our nanoparticles act inside neutrophils, and intracellular H_2O_2 concentration is expected to be higher than extracellular H_2O_2 concentration. Therefore, we used H_2O_2 concentrations of greater than 65 μM, specifically, 100 and 200 μM in Fig. 2f.

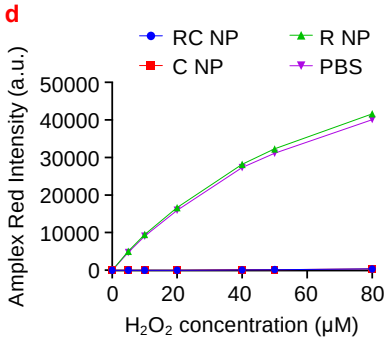


Fig. 2d Remaining amount (Amplex Red intensity) of H_2O_2 after reaction with nanoparticles at various H_2O_2 concentrations (n = 3).

[Results] (Page 6, Line 100): Catalase loaded in RC NPs reacted with H_2O_2 (Fig. 2d) and produced oxygen (Fig. 2e).

[Methods] (Page 22, Line 441): Roscovitine release was evaluated by dialyzing (molecular weight cutoff 3000) RC NPs in 35 ml of PBS with or without 100 or 200 μM H_2O_2 and incubating samples at 37°C with shaking (100 rpm).

[Methods] (Page 22, Line 444): The Amplex™ Red Hydrogen Peroxide/Peroxidase Assay Kit (Thermo Fisher) was used to determine the amount of H_2O_2 remaining after reaction with nanoparticles. 50 μL of H_2O_2 (diluted in 1x reaction buffer) was mixed with 10 μL of nanoparticles (or PBS) and incubated at room temperature for 5 minutes before addition of 50 μL of working solution consisting of Amplex™ Red Reagent and horseradish peroxidase. Fluorescence was

measured using a fluorescence microplate reader. Amplex Red intensity values were subtracted from average intensity values at 0 μM H_2O_2.

2, The author stated that "As RC NPs release roscovitine slowly in unactivated neutrophils and non-ROS conditions (Fig. 2E)". The stability of RC NPs over a 7-day period is evident from Fig. 2C. Interestingly, within the first 12 hours, the RC NP+H2O2 group demonstrates a 60% release of Roscovitine, while other groups exhibit a release rate of over 30%, indicating a moderate but not slow release profile (Fig. 2E). This rapid initial release could be attributed to the degradation of PLGA. However, if this is not the case, it would be essential to explore alternative mechanisms for Roscovitine release, aside from the typical burst release associated with RC NPs.

Response 2: We thank the reviewer for drawing our attention to this matter. Firstly, we have edited the statement "As RC NPs release roscovitine slowly in unactivated neutrophils in non-ROS conditions (Fig. 2e)" to "As RC NPs release roscovitine in unactivated neutrophils and non-H_2O_2 conditions more slowly than in H_2O_2 conditions (Fig. 2f) (Page 19, Line 397)" to refrain from describing the drug release as slow.

We attribute the burst release of roscovitine from PLGA NPs in the initial period (first 1-2 days) to a combination of degradation and diffusion, as suggested by numerous studies[28 29, 30, 31, 32]. Mass loss of PLGA nanoparticles is known to initiate within the first two days after incubation in water as demonstrated by previous studies[33, 34, 35]. Diffusion of drug also occurs immediately after incubation of NPs in water as drug embedded near the surface of PLGA nanoparticles is quickly released into the surrounding solution.

As for the size stability of RC NPs, previous studies have shown that degradation of PLGA nanoparticles does not significantly affect nanoparticle size for the first several weeks[29, 36]. Specifically, in a previous study, the size of 150 nm PLGA particles was maintained for up to three weeks[29]. These results are in line with our size stability results in Fig. 2c. Therefore, we believe that NP size stability does not imply that there is no degradation of PLGA.

In conclusion, a combination of (1) degradation and (2) diffusion can explain the initial burst release of 30% in the first 12-24 hours in "normal PLGA NPs". In H_2O_2-treated RC NPs, a combination of (1) degradation, (2) diffusion, and (3) morphological deformation of the NPs contributes to the enhanced rate of drug release.

We thank the reviewer for drawing our attention to this matter, and we have now included these important points in the results section of our manuscript.

[Results] (Page 7, Line 114): All NPs displayed over 30% roscovitine release within the first 12 to 24 hours (Fig. 2f). The initial burst release of roscovitine in the R NP groups and the non H_2O_2-treated RC NP group can be attributed to a combination of PLGA degradation and drug diffusion, which initiate in the first few days after incubation in water[33, 34, 35 28 29, 30, 31, 32]. The enhanced rate of drug release in the H_2O_2-treated RC NP groups can be attributed to morphological deformation (Fig. 2g) in addition to PLGA degradation and drug diffusion.

3, As observed in Fig. 2G, RC NPs appear to be predominantly localized on the membrane of neutrophils.

Response 3: We sincerely thank the reviewer for drawing our attention to this matter. We have revised our data in Fig. 2g (now Fig. 2h) with images that more accurately depict the internalization of RC NPs. In our revised data, we conducted confocal microscopy at two hours instead of one hour in our previous experiment, which we believe may play a part in showing better RC NP internalization.

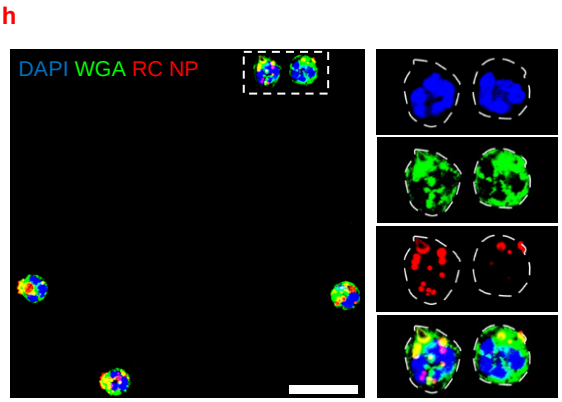


Fig. 2**h** RC NP uptake by neutrophils as visualized by confocal microscopy. Scale bar, 20 µm. WGA stains for cell membrane. Dotted lines show edge of cell.

[Results] (Page 7, Line 125): RC NP internalization was verified by confocal microscopy after two hours (Fig. 2h).

[Methods] (Page 23, Line 477): Isolated neutrophils were treated with RC NPs containing 20 µM roscovitine and internalization was assessed one hour later by flow cytometry and two hours later by confocal microscopy.

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REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

Authors are responsive to the previous comments, thus the manuscript improved significantly for scientific clarity of data interpretation.

Reviewer #2 (Remarks to the Author):

The author has addressed my concerns and revised the manuscript accordingly. I am now satisfied with the revisions and recommend acceptance.

RESPONSE TO REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Authors are responsive to the previous comments, thus the manuscript improved significantly for scientific clarity of data interpretation.

Response: We thank the reviewer for their constructive comments throughout the revision process. We agree that the reviewer's input has significantly improved the quality of our manuscript.

Reviewer #2 (Remarks to the Author):

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Response: We thank the reviewer for their constructive comments throughout the revision process. We believe that the reviewer's input has significantly improved the quality of our manuscript.