ROUND 1

RE：Manuscript NO.: 67776
Title: Exosomes derived from inflammatory myoblasts promote M1 polarization and break the balance of myoblast proliferation/differentiation

EDITORIAL OFFICE'S COMMENTS
(1) Science editor: Recommend for rejection. Scientific classification: Grade B, Grade E, and Grade B. Language classification: Grade A, Grade B, and Grade C. Scientific classification does not meet the publication standard of WJSC. Reviewer pointed out that the article is well designed, original. However, the picture information has not been fully discussed. Several experimental methods were not described in detail in this paper, and the contents of mesenchymal stem cells unrelated to this paper appeared in many parts. Therefore, I suggest reject this manuscript.
Response to science editor: Thank you for your comments concerning our manuscript. We have carefully revised the wrong contents about mesenchymal stem cells unrelated to this paper throughout the whole manuscript. Besides, we added more experimental details in the Methods section and discussed the picture information as much as we can. We hope after those revisions, our work can meet the publication standard of WJSC.

(2) Company editor-in-chief: I have reviewed the Peer-Review Report, the full text of the manuscript, and the relevant ethics documents, all of which have met the basic publishing requirements of the World Journal of Stem Cells, and the manuscript is conditionally accepted. I have sent the manuscript to the author(s) for its revision according to the Peer-Review Report, Editorial Office's comments and the Criteria for Manuscript Revision by Authors. However, the quality of the English language of the manuscript does not meet the requirements of the journal. Before final acceptance, the author(s) must provide the English Language Certificate issued by a professional English language editing company. Please visit the following website for the professional English language editing companies we recommend: https://www.wjgnet.com/bpg/gerinfo/240.

Response to company editor-in-chief: Thank you for your letter and the reviewers' comments concerning our manuscript. These comments have been valuable in ensuring we produce a high-quality manuscript and have also been informative in guiding our research. We have reviewed the comments carefully and have made corrections with the hope that they meet the reviewers' standards. We have answered the reviewer's comments carefully and step by step as follows. Revised portions are marked in **blue** throughout the paper, and changes to the manuscript are also highlighted with **red** text. These changes have substantially improved our manuscript while preserving the content and general framework. We have also re-sent our article to a professional English language editing service again to improve its readability.
We appreciate the reviewers' and editor's careful review of our work and thank you

for your comments and suggestions.

Reviewer #1:

Specific Comments to Authors: In this paper, the authors evaluated the effects of exosomes from inflammatory C2C12 myoblast on macrophage polarization and myoblast proliferation/differentiation. However, several experimental methods were not described in detail in this paper, and the contents of mesenchymal stem cells unrelated to this paper appeared in many parts. Please review your paper carefully and submit it after revision.

Response to reviewer 1: Thank you for your kind comments, we have carefully revised the manuscript according to your comments and added additional experimental details. Below are some examples that respond to your questions.

For example,

"The NTA experiment evaluated the size of the C2C12-Exos."

"The labeled PBS or C2C12-Exos were co-incubated with C2C12 cells and macrophages (M0) for 12 h in a 37° C, 5% CO2 cell incubator."

"0.5 ml of penicillin/streptomycin solution were used to culture C2C12s for 48 h."

"The morphology of C2C12-Exos was observed by transmission electron microscopy (left) and scanning electron microscope (right). The red arrows point to representative exosomes."

"***Western blot***

Protein was extracted and analyzed using an established method[1]. Briefly, the total protein from C2C12 was collected by RIPA lysis buffer (R0010; Solarbio, Beijing, China) with Phenylmethanesulfonyl fluoride (PMSF; Solarbio, Beijing, China). The BCA Protein Assay Kit (Beyotime Biotechnology, Shanghai, China) was used to measure the concentration of protein. Next, 10 μg protein samples from each group were separated by 10% SDS-PAGE. After that, they transferred to nitrocellulose membranes. 5% non-fat milk dissolved in Tris-buffered saline containing Tween-20 was utilized to block the blots before applying primary antibodies overnight at 4 ◦C. Anti-CD206, anti-Arginase 1, anti-iNOS, Anti-CD86, anti-MYHC, anti-MyoD1, anti-MyoG, anti-Col 1, anti-α-SM, anti-β-Tubulin, and anti-GAPDH antibodies were applied as primary antibodies (Table 1). Each group contains 4 protein samples for calculation (n=4). "

"***Flow Cytometry for M1/M2***

Flow cytometry was performed using anti-CD86-PE, anti-CD163-APC, and anti-F4/80-FITC (Thermo/eBio). The percent of CD86 + / CD163 + / F4/80 + cell population (macrophage) was evaluated using Cytomics™ FC 500 (Beckman Coulter). In detail, the macrophages from different groups were collected with flow cytometry staining buffer (ebioscience). Then, 2 ul antibody was added to every 100 ul cell suspension for 60 mins at 4℃ in darkness. After then, 5ml staining buffer was put into each tube and the cell suspension was centrifuged for 5 mins (500g, 4℃). The washing procedures were repeated three times. Last, the cell precipitation was re-suspended by 200 ul PBS to wait for flow cytometry analysis."

"Enzyme-linked immunosorbent assay (ELISA).

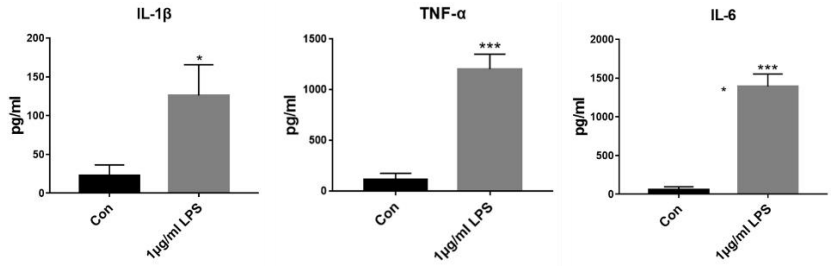
ELISA kits, including IL-6, IL-1β, TGF-β, and TNF-α, were purchased from Laizee

(LEM060-2, LEM012-2, LEM822-2, LEM810-2). The protocol was according to the previous study[33]. Cell supernatant of each group was collected, and then those kits were utilized following the manufacturer's instructions. In detail, add 100 ul sample and standard sample to the corresponding hole, seal the plate, and incubate at room temperature for 2 hours. Then, discard the sample, wash the plate with 300 ul lotion 5 times, and drain on paper. After then, add 100 ul avidin HRP solution to each well, seal the plate, and incubate at room temperature for 30 mins. Repeat the washing procedure. Next, add 100 ul TMB solution to each hole, seal the plate, and incubate 15min at room temperature. Last, add 50ul terminator to terminate the reaction and the plate was analyzed at 450nm."

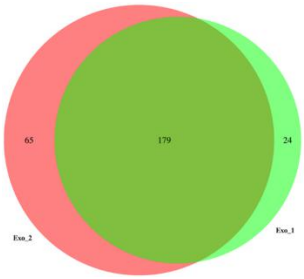
Reviewer #2:
Specific Comments to Authors: Reviewer Comments Name of Journal: World Journal of Stem Cells Manuscript NO: Manuscript Type: ORIGINAL ARTICLE Basic Study Exosomes derived from inflammatory myoblasts promote M1 polarization and break the balance of myoblast proliferation/differentiation Zhiwen Luo, Yaying Sun, Jinrong Lin, Beijie Qi, and Jiwu Chen Summary: The start with the premise that excessive inflammation leads to fibrosis, but this fibrosis could be controlled by reducing macrophage polarization using exosomes generated from muscle cells (C2C12). The C2C12 Exos induce M1 macrophage which is proinflammatory and this inflammatory environment leads to suppression of myoblast differentiation, but increases proliferation thereby leading to muscle regeneration and prevent fibrosis. It seems paradoxical that inflammation that started the process itself helps in muscle regeneration and when its been know that prolonged inflammation leads to fibrosis.
Response to reviewer 2: Thank you very much for your positive feedback and meaningful suggestions for our manuscript, below are the point-by-point responses to each of your comments.

Q1: The first thing authors wish to recreate, is the inflammatory environment that muscle cells experience during injury. To achieve this authors, used LPS, but its not shown whether LPS concentration has induced changes in the C2C12 cells. LPS addition ideally inhibit differentiation of C2C12, however this has not been demonstrated, So, its is not clear if LPS treated C2C12 cells are inflamed and are now ready for harvesting exosomes.
Response 1: Thanks for your kind advice! LPS was widely used to induce inflammatory responses in both animal and cell models.[2–6] It was also accepted in many C2C12-related studies.[7–14] For example, Park et al. treated the C2C12-myoblast with 1 mg/ml LPS for 1d and found that both the TNF-α, IL-6, and related inflammatory pathway proteins significantly increased.[14] Therefore, our work was based on those findings, including treatment concentration and time. But, to answer your question, we performed the Elisa experiment to verify if the LPS treated C2C12 cells are inflamed, including IL-1β, TNF-α, and IL-6. The results showed that IL-1β, TNF-α, and IL-6 protein levels significantly increased after LPS treatment for 1d. Furthermore, our study is a series of works that we further performed the miRNA

sequencing on the exosomal miRNA between IF-C2C12-Exos and normal C2C12-Exos. We found that many inflammatory miRNAs were significantly up-regulated and anti-inflammatory miRNAs were correspondingly down-regulated. We hope those results can solve your confusion. We will continue to carry out further works on myoblasts. Once again, thanks for your suggestion. Some of those data were shown below.



Elisa results

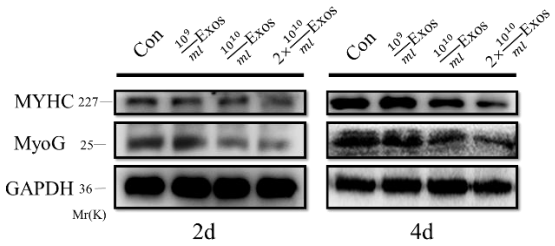


Venn Diagram for miRNAs of IF-C2C12-Exos and normal C2C12-Exos

Q2: The authors using immunolocalization showed how Exos affect myofibroblast differentiation but I would advise the authors to perform western blotting to quantitate these changes between different exosome doses. Although authors in Figure 9 show western blot data, but even 24hrs seems sufficient to induce same changes that 2 and 4 days have given. It seems surprising that these changes can occur after 24 hrs. And authors should address this in the discussion.

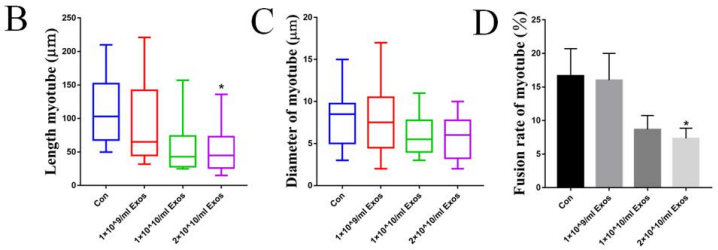
Response 2: Thanks for your advice. The myoblast differentiation is a continuous process, in other words, the changes or delay caused by IF-C2C12-Exos would influence the following differentiation process and the whole process would be slow down.[15] That's why the immunolocalization and WB results showed that 24hrs seems sufficient to induce the same changes that 2 and 4 days have given. Besides, the western blot results in many myoblast-related studies were just performed immediately after treatment because after the stimulation was removed, the cells would try to come back to the normal level.[2–6] That is the reason why under the condition of low-level of the myogenic proteins (MyoD, MyoG, and MYHC) after 1d-IF-C2C12-Exos treatment, the myogenic differentiation still occurred and was showed by immunolocalization after 2 and 4 days. However, to solve your question, we conducted the western blot experiment to quantitate the changes after different concentrations of IF-C2C12-Exos treatment for 2/4 days and the results were shown below. The total level of MyoG and MYHC protein was up-regulated with time increasing, which is consistent with the immunolocalization results. In addition, with

the concentration of IF-C2C12-Exos increasing, both MyoG and MYHC protein levels were down-regulated compared with that of normal myogenic C2C12 at the same time point. We hope those results can answer your question.

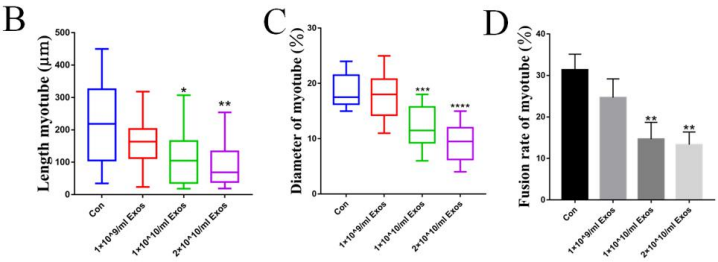


Q3: The giemsa stained myotubes has been shown after 2 days and 4 days of IF C2C12 Exos. But there appears to be no difference between day 2 and day4 treatment. There is no explanation offered by the authors.

Response 3: Thanks for your query. Under normal conditions, the myogenic process can be primarily shown on day 2 and be clearly presented on day 4 by immunolocalization.[16–18] That's to say that the primary aims of experiments at each time point were different, that's why we performed this experiment on both day 2 and day 4. Besides, there were indeed differences between 2 days and 4 days of IF-C2C12-Exos treatment. For example, on day 2, there was no difference in the mean diameter of myotubes among the four groups, but the significant reduction occurred in the high concentration IF-C2C12-Exos group on day 4. In addition, the differences in the fusion rate and length of myotube also became greater on day 4 than that on day 2.



Day 2



Day4

Q4: In the Figure 2E, it is not indicated what the 4 lanes are? Are they duplicates?

Response 4: Thanks for your question. It is not repeated samples, but they are different exosomal proteins and deproteinized supernatants extracted from the two

independent conditional mediums. To avoid misunderstanding, we added a description in the corresponding figure legend as follows.

"The four lanes represented different exosomal proteins and deproteinized supernatants extracted from the two independent conditional mediums."

Q5: Scale bars not shown for all images. Scale bars should be shown for every IF, geimsa stained and brighfield image.

Response 5: This is a helpful suggestion. We checked all the figures and added the corresponding scale bars. The revised figures were shown below.

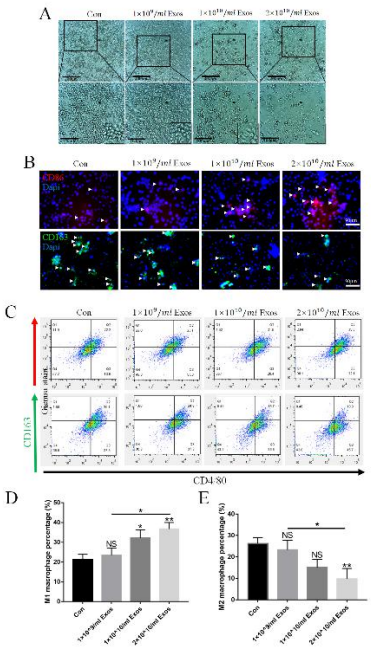


Fig. 4

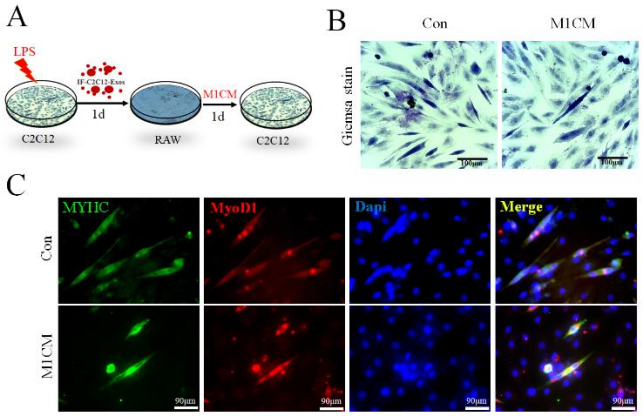


Fig. S2

Q6: It is not clear how the conditioned media for C2C12 was collected. Especially since author mention " The C2C12 conditioned mediums (C2C12-CM) were collected after 24h and the exosomes from C2C12 myoblast (C2C12-Exos) were extracted by following steps. RAW 264.7 cells (mouse leukemia cells of monocyte-macrophage), purchased from the American Type Culture Collection, were maintained in DMEM with 10% FBS and 0.5 ml of penicillin/streptomycin solution in a humidified incubator at 37°C and 5% CO2 atmosphere. I request the authors to clear this procedure.

Response 6: Thanks for your kind suggestion. We added details of this procedure as follows.

"The C2C12 conditioned mediums (C2C12-CM) were collected by a 1 ml Pipette and were added into 50 ml tubes after 24h incubation. Then, the mediums were kept in -80℃ before using. The exosomes from C2C12 myoblast (C2C12-Exos) were extracted by following steps. "

Q7: There are some jarring words, which do not seem appropriate (highlighted in bold), for

example

- The Lipopolysaccharide, LPS, with a concentration of 1000ng/ml was used to induce the inflammatory environment for C2C12. Then, washed the medium three times to **abandon** LPS and a fresh exosome-depleted medium was added.
- **The procedure of SEM referred to the previous study**.
- Generally, **freeze** 100 μl of the exosome suspension overnight in the refrigerator and transferred it to a vacuum dryer for lyophilization.
- Then, the sample was coated with gold by an ion sputterer and observed **in the microscope**.
- **Detailly,** the decline of MyoD, MyoG, and MYHC protein levels suggested that IF-C2C12-Exos evidently reduced the myogenic differentiation ability of myoblast.

Response 7: Thanks very much for pointing out those mistakes. We revised those words according to your advice. The followings were the revised version.
"Then, washed the medium three times to clear all the LPS and a fresh exosome-depleted medium was added."
"The procedures of SEM were based on the previous study"
"Generally, 100 μl of the exosome suspension were frozen overnight in the refrigerator and transferred to a vacuum dryer for lyophilization."
"Then, the sample was coated with gold by an ion sputterer and observed under the microscope."
"In detail, the decline of MyoD, MyoG, and MYHC protein levels suggested that IF-C2C12-Exos evidently reduced the myogenic differentiation ability of myoblast."

Reviewer #3:
Scientific Quality: Grade B (Very good)
Language Quality: Grade A (Priority publishing)
Conclusion: Accept (High priority)
Specific Comments to Authors: The article is well designed, original, and I would love to recommend it for publication.
Response to reviewer 3: Thank you very much for your positive comments and praise for our study. Our team will continue to contribute more interesting works in the field of stem cells.

Once again, we sincerely thank all the three reviewers very much for their wonderful suggestions!

ROUND 2
RE：Manuscript NO.: 67776
Title: Exosomes derived from inflammatory myoblasts promote M1 polarization and break the balance of myoblast proliferation/differentiation

EDITORIAL OFFICE'S COMMENTS

(1) Science editor Fan: 1. There are some specific comments to be modified in the second-round review. Please revise the manuscript according to its comments and make a point-to-point response to the review comments （67776_RevisionReviewReport）. ----2. Please provide all 2 fund documents （the project of the National Natural Science Foundation of China，grant number 81772419; 81972062）. ----3. Please mark the position of Figure 10 in the manuscript （67776_Auto_Edited）. ----4. Please rearrange the figure. If one figure cannot fit, please enlarge the size of the PPT, or put Figure 1 in several slides respectively. Please ensure that the PPT size is less than 10MB. Please refer to my modified Figure 2. -----5. All modifications to the contents of the manuscript should be completed on the basis of the attached manuscript （67776_Auto_Edited）.

Response to Science editor Fan:

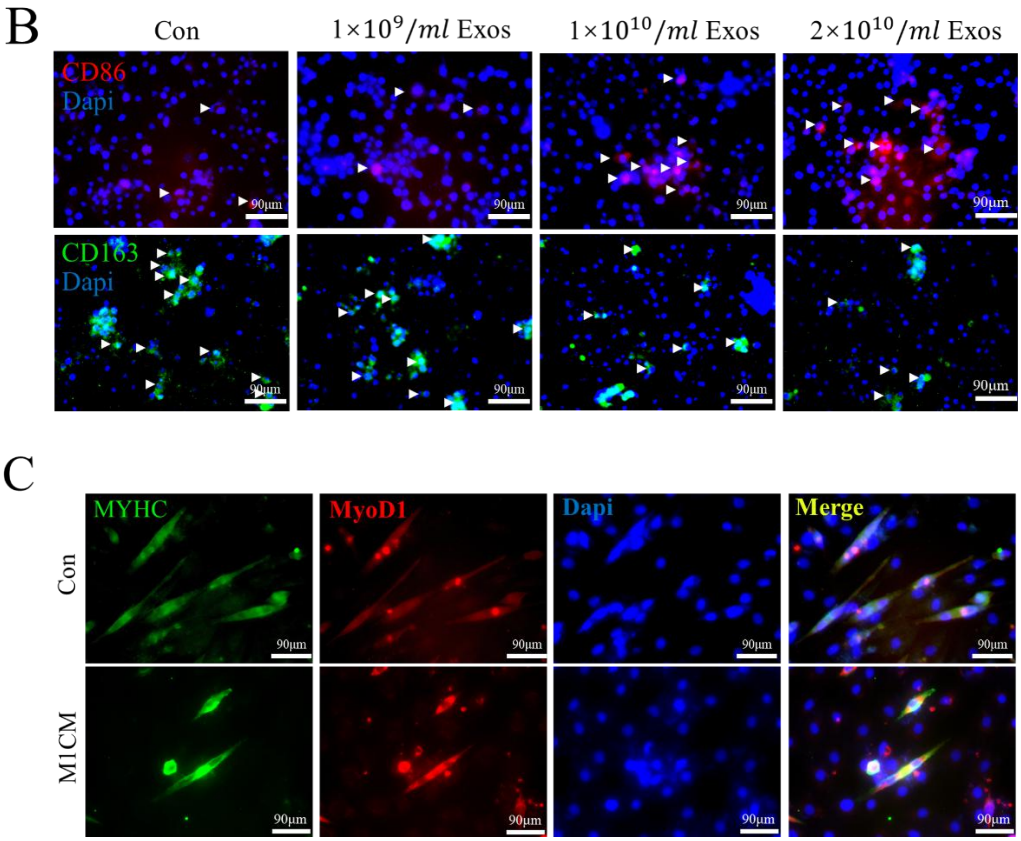
Thanks for your comments, we have carefully prepared the manuscript according to your comments. 1. We replied the reviewer point-to-point response and revised it carefully. 2. The files were uploaded. 3. We marked it in the manuscript. 4. We have separated all the figures to fit the PPT scale according to your requirements. Although we tried our best to reduce the size of figures, the PPT is still larger than 10MB. We hope you can understand the difficulty.5. All the modifications were completed based on the attached manuscript （67776_Auto_Edited）.

Reviewer #1:

The authors have asnwered most of my queries effectively. However, there are some revisions necessary. The authors have claimed that scare bars have been added to all images as per my suggestion. But if i see the powerpoint file uploaded by the authors the scale bars are only sporadically added. Also, in material and methods section authors use 'uL"to denote micro litres, but this is not correct convention. The authors must use greek alphabet micro instead of english u. Also, the authors havent highlighted the changed done in the revised main text, so its difficult to verify the claims made by authors.

Response to reviewer 1: Thank you for your kind comments, we have carefully revised the manuscript according to your comments, including micro litres in material and methods section. In addition, the changed revisions were shown in red in our manuscript.

Besides, scale bars were added to all the figures as follows:


B
Con
1×10^9/ml Exos
1×10^10/ml Exos
2×10^10/ml Exos
CD86
Dapi
CD163
Dapi
90μm
C
MYHC
MyoD1
Dapi
Merge
Con
M1CM
90μm