

Responses to editors and reviewers

Manuscript title: Etanercept restores the matrix formation of hADSCs in the late stage of chondrogenic differentiation

Dear Editors and Reviewers:

Thank you for your letter and the reviewers' comments concerning our manuscript "Etanercept restores the matrix formation of hADSCs in the late stage of chondrogenic differentiation". All your valuable comments are very helpful for revising and improving our manuscript. We have already studied the comments and made our best efforts to rectify the article paper. Therefore, we are very hopeful for your kind approval in this regard. Revised portions are marked red in the paper. The main corrections and the response to the review's comments are mentioned below.

Response to reviewer's comments:

Reviewer #1:

Comment: 1) Besides etanercept, another one of the TNF- α inhibitors should be used to investigate the effect on the chondrogenic differentiation and the modulation of TNF- α in the NF- κ B pathways. And so did at least the two dosages of TNF- α inhibitors.

Author's response: I have already performed the additional experiments with another TNF- α inhibitors (infliximab, 10 μ g/ml). And the dosages of TNF- α inhibitors was set based on the instruction of the product and existing researches:

1. Bibikova, E.; Youn, M. Y.; Danilova, N.; Ono-Uruga, Y.; Konto-Ghiorgi, Y.; Ochoa, R.; Narla, A.; Glader, B.; Lin, S.; Sakamoto, K. M., TNF-mediated inflammation represses GATA1 and activates p38 MAP kinase in RPS19-deficient hematopoietic progenitors. *Blood* 2014, 124 (25), 3791-8.
2. Balzano, T.; Dadsetan, S.; Forteza, J.; Cabrera-Pastor, A.; Taoro-Gonzalez, L.; Malaguarnera, M.; Gil-Perotin, S.; Cubas-Nunez, L.; Casanova, B.; Castro-Quintas, A.; Ponce-Mora, A.; Arenas, Y. M.; Leone, P.; Erceg, S.; Llansola, M.; Felipo, V., Chronic hyperammonemia induces peripheral inflammation that leads to cognitive impairment in rats: Reversed by anti-TNF-alpha treatment. *J Hepatol* 2020, 73 (3), 582-592.

Comment: 2) The effect of TNF- α inhibitors on the expression of NF- κ B and MMP3 proteins in the control medium for chondrogenic differentiation of hADSCs should be studied and compared with the other groups.

Author's response: The control group was hADSCs cultured in growth medium containing 10% FBS and 1% antibiotics for 7 days before harvest. The GM group of the WB were ADSCs harvested above unless otherwise marked.

Because we believe that ADSCs cultured in vitro for 7 days could retain good cell status and protein secretion ability. In the additional experiments, we collected the protein sample of GM group on Day 14 and Day 28 and performed western blot test.

Comment: 3) The statistical analysis should be described more clearly as one to one correspondence.

Author's response: I have already quantified the blots in the Figures and analyzed the data with on sample T test. However, due to the different exposure duration of different samples, the variance of quantified data obtained was too large, and the P value of many bands was too large when the variation trend was in line with the expectation

Comment: 4) In Figure2, what were the changes in Aggrecan and COL2 at 14d when compared with control or those at 7d? The similar problem was also found in Figure 4a.

Author's response: After quantifying the bands, it could be seen that the expression of Aggrecan and COL-2 on the day 14 was higher than that on the day 7 and the control group.

Comment: 5) In Figure3a, the expression of MMP3 in GM group was at 7d or 14d? In this figure, the control group should be set at 7d and 14d respectively.

Author's response: In Figure3a, the expression of MMP3 in GM group was at 7d. We collected the protein sample of GM group on Day 14 and Day 28 and performed western blot test (Figure 5c). But we have ran out of the samples which were collected from hADSCs treated with Etanercept. I'm sorry we won't be able to repeat this comparison anytime soon.

Comment: 6) In Figure 5, semi quantitative WB analysis of MMP3 and NF-kB should be carried out.

Author's response: Thank you for the suggestion, we have already carried out semi quantitative WB analysis of MMP3 and NF-kB in Figure 5.

Comment: 7) The author presented that IL-1 β alone did not affect the expression of TNF- α or MMP-3 in hADSCs while it upregulated the expression of MMP-3 in the presence of a chondrogenic differentiation medium. These results should be discussed in detail.

Author's response: In this part of the study, the effect of IL-1 β on the cell viability of ADSCs was great, so the observation was not carried out for more than 7 days. This part of the research results will be better designed and further

discussed in future research. We are very sorry that these results in only for presentation. We hope it has little impact on the core conclusion of the paper.

Reviewer #2:

Comment: 1) Data from the following studies provides a broader perspective of inflammatory mechanism and treatments and should be integrated in the introduction: PMID: 29959408, PMID: 35568708, PMID: 17151319, PMID: 17151316, <http://dx.doi.org/10.4236/jdm.2011.13006>, PMID: 15362483. It could have been interesting to compare the current results with the effects of other inhibitor that does not target TNF.

Author's response: Thank you for providing the studies, we have read the articles mentioned above and discussed them in the part of introduction of the revised manuscript.

Comment: 2) Proofreading is required.

Author's response: We have sent the revised manuscript to AJE the get the language polished after finishing the additional experiments.

Comment: 3) Who exactly has waived “patient consent”?

Author's response: The ADSCs was obtained from the adipose tissue of

the patients who went through liposuction. The information recorded with the sample only included the gender and age, so that it was impossible to identify the patients. For the reasons above, the Ethics Committee of science study, Peking university Shenzhen hospital has waived “patient consent” and the Ethics Record Number was 2022-076.

Comment: 4) The abstract is not coherent and must be re-written using shorter and more clear sentences. As most of the “core tip” is a copy/paste from the abstract, the core tip must be rephrased too and preferably be reworded differently from the abstract.

Author's response: Thanks for the suggestion, we have already re-written the core tip and the abstract.

Comment: 5) What is meant by “P3 hADSCs”? All abbreviations must be reviewed.

Author's response: “P3 hADSCs” means the ADSCs obtained from human and was cultured to passage 3. And we have rectified the description of “P3 hADSCs” with “Passage 3 hADSCs”.

Comment: 6) In figure 1, were those cells “P3 hADSCs”, P1 or P2? This

need to be clarified and clearly stated in the manuscript.

Author's response: All of the experiments were performed with Passage 3 hADSCs to make it more comparable and convincing.

Comment: 7) How many times have all presented experiments been repeated?

Author's response: As for the identification of ADSCs, the western blot and flow cytometry was performed only one time. As the rest of the experiments, the western blot was performed 3 times with 3 different sample. The images of toluidine blue staining and immunofluorescent staining was selected from 5 images five different visual fields of each sample.

Comment: 8) Fig 1c need to be better labelled and be consistent with data presented in the result section.

Author's response: We have replaced the image of alizarin red staining in Fig 1c to make them in accordance with the result section.

Comment: 9) All western blots need to be quantified and their uncropped gels added as supplementary data.

Author's response: We have quantified the western blots and presented

them in each figure. Due to different sample was tested, the variance of WB quantitative results was large, so some data had excessive P-values when the trend was in line with expectations. In revised figures, we only presented the representative blots and other blots was provided in the supplementary figures. We have provided the pictures of uncropped gels of most of the western blots, due to the longtime scale of this research and different machine of chemiluminescence was used, some of the uncropped gels was not photographed.

Comment: 10) Quantify the scratch assay in fig 2c. The list of references needs to be diversified and more inclusive.

Author's response: We have quantified the scratch assay in fig 2c. And we have read more articles related and discussed them in the revised manuscript.

Comment: 11) If considered, the following studies would help addressing that concern and provide more insights into molecular bases of anti-inflammatory and proapoptotic mechanisms: PMID: 34662244, PMID: 35211395, PMID: 35517830, PMID: 35740022, PMID: 35177980, PMID: 33255507, <https://doi.org/10.1039/D0NA00958J>, [10.1097/HM9.0000000000000008](https://doi.org/10.1097/HM9.0000000000000008), <https://doi.org/10.1186/s41936-020-00177-9>, <https://doi.org/10.1186/s41936-021-00251-w>.

Author's response: Thankyou for providing the studies, we have read the articles mentioned above and discussed them in the part of discussion and conclusion of the revised manuscript.