Peer Review File

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Reviewer A

Comments to the Author

In this study, Liu and colleagues assess the therapeutic effect of human umbilical cord mesenchymal stem cells (UC-MSCs) in murine acute liver failure (ALF), using the D-GalN/LPS ALF model. They describe that pre-treatment of mice with UC-MSCs (prior to induction of ALF) reduces indices of liver injury (e.g., serum ALT/AST, liver damage) by inhibiting apoptosis, inflammation and pyroptosis. These are interesting findings, with regards to those aspects, however there are some limitations in this study. Please see below for some comments/suggestions

Comment 1: More emphasis should be given on UC-MSCs' current utility/potential in liver disease (in Introduction), and how this study's results build on previous findings in primate ALF research with UC-MSCs, and what they offer for future research (in Discussion)

Reply 1: Thanks for your suggestion. We have added description and references in the section of Introduction and Discussion.

Changes in the text: please see page 5, line 87-91; Page 18-19, line 386-387.

Comment 2: The ameliorating effects of UC-MSCs on the ALF-related inflammation, apoptosis and pyroptosis have been investigated only prior to induction of ALF (administration of UC-MSCs 1h before D-GalN/LPS injection). Therefore, to assess their therapeutic effect/ potential, i.v. administration of UC-MSCs at latter points following ALF (e.g., at 6h or 24h after i.p. injection of D-GalN/LPS) should be examined, and then measure ALT levels, liver necrosis, pyroptosis etc.

Reply 2: As suggested, we conducted the animal experiment about the administration of UC-MSCs at 6h after i.p. injection of D-GalN/LPS. Hepatic histology, serum aminotransferases, and liver inflammation were examined. The results indicated that UC-MSC treatment obviously ameliorated D-GalN/LPS-induced acute liver injury.

Changes in the text: We added the methods in Page 7, line 135-137; the results in page 13, line 259-263, line 270-271, page 14, line 290-292; and the data in supplementary Fig. 1.

Comment 3: In the Raw264.7 co-culture with UC-MSCs-CM experiment it is unclear which medium is used in the "LPS" group: is it DMEM as per control? or same CM used for the culture of UC-MSCs? Also, were Raw264.7 cultured in the presence of 100% UC-MSCs-CM, or mixed with DMEM? It would be good to comment if the results observed are cytokine-mediated and/or exosome-mediated (e.g., exo contained in UC-MSCs-CM)

Reply 3: DMEM was used in LPS group, and 20% DMEM + 80% UC-MSCs-CM was used in UC-MSCs+LPS group. We have modified the description in the revised manuscript.

We agree with the reviewer's comments. Indeed, cytokine and/or exosome may play an important role in the therapeutic effects of UC-MSCs-CM. We have extended the discussion about this issue. The exact mechanisms will be considered in our future studies.

Changes in the text: We revised the description in page 11, line 222-223 and extended the discussion in page 18, line 365-369.

Comment 4: Given the crucial role of F4/80+ macrophages (Tim-4+ Clec4F+ liver-resident Kupffer cells and Clec4F- monocyte-derived macrophages) in the immunopathology of ALF, it would be good to examine each subset's numbers by IHC/IF e.g., Clec4F. Does UC-MSC pre-treatment of mice reduce recruitment of monocyte-derived macrophages? (thus, reducing liver injury/severity)

Reply 4: Thanks for the sincere comments. Previous studies (Ref. 1-3) indicated that CD11b was an important indicator of monocyte-derived macrophage infiltration in acute liver injury. We have added the experiment about $CD11b^+$ immunofluorescence staining of the liver.

Ref.1. Lu L, Yue S, Jiang L, et al. Myeloid Notch1 deficiency activates the RhoA/ROCK pathway and aggravates hepatocellular damage in mouse ischemic livers. Hepatology 2018; 67:1041-1055.

Ref.2. Jin Y, Li C, Xu D, et al. Jagged1-mediated myeloid Notch1 signaling activates HSF1/Snail and controls NLRP3 inflammasome activation in liver inflammatory injury. Cell Mol Immunol 2020; 17:1245-1256.

Ref.3. Li C, Sheng M, Lin Y, et al. Functional crosstalk between myeloid Foxo1-β-catenin axis and Hedgehog/Gli1 signaling in oxidative stress response. Cell Death Differ 2021; 28:1705-1719.

Changes in the text: We have added $CD11b^+$ immunofluorescence staining experiment, please see Fig. 3B and page 14, line 288.

Comment 5: - Results/Figures -

The resolution of few images is low, therefore some graphs incorporated in figures are blurry and difficult to read (e.g., 1B, 1E, 3A, 3B). Please ensure to revise.

Reply 5: We are sorry for the blurry images and have revised the resolution of images.

Changes in the text: Please see all the figures.

Comment 6:

Fig. 1F: in addition to representative H&E staining images, quantification of necrosis (e.g., % area of necrosis) should be provided for the different groups assessed.

Reply 6: As suggested, the quantification of necrosis in Fig. 1F has been assessed.

Changes in the text: Please see the Fig 1F.

Comment 7: Fig. 5A-B: the X axis' graph labels should be more clear, "LPS" vs "UC-MSCs-CM plus LPS", as per experimental set-up

Reply 7: The X axis' graph labels have been revised to "LPS", UC-MSCs-CM+LPS.

Changes in the text: We have revised the X axis' graph labels as advised in Fig 5A, B.

Reviewer B

Comments to the Author

The authors presented a study showing that human umbilical cord mesenchymal stem cells are a potential therapeutical tool to prevent inflammation in an animal model of acute liver failure. However, there are some major comments that this reviewer has for the authors:

Comment 1: Acute liver failure is mostly produced by viral infections and drug toxicity, as the authors clearly stated in the first paragraph of introduction. However, their inflammatory model is based on a bacterial-like infection using LPS. Their argument is only based on the morphological features LPS induces similar to virus. However, the immune response exerted, and the cytokine cocktail activated by virus is significantly different than the one induced by bacterial infection. Therefore, their findings about the use of UC-MSCs can only be applied in cases of acute bacterial infection of the liver, but not other etiologies like viruses or drug toxicity (the most common causes of ALF), unless proven.

Reply 1: Thanks for your thoughtful comments and suggestions. We agreed that D-GalN/LPS induced ALF was based on a bacterial-like infection. Indeed, this is a limitation of our current study. We added the limitation in the section of Discussion. We also revised the description about liver features induced by D-GalN/LPS in the section of Introduction. The effects of UC-MSCs on ALF induced by viruses or drug will be taken into consideration in our future studies.

Changes in the text: Please see page 4, line 68-69 and page 16, line 340-341.

Comment 2: The authors administered the UC-MSC one hour before the LPS/D-GalN injection. Therefore, UC-MSC are preventing the development of inflammation as a prophylactic therapy, instead of a reversion of the inflammation already established by the stressor. The authors must consider that, in line 331 of their discussion, UC-MSC did not improved liver function, but instead protected it, or prevented significant damage.

Reply 2: Thanks for the valuable comments. We have added the animal experiment about the administration of UC-MSCs at 6h after i.p. injection of D-GalN/LPS. Our results indicated that after D-GalN/LPS injection, UC-MSC treatment significantly attenuated D-GalN/LPS-induced acute liver injury.

Changes in the text: We added the methods in Page 7, line 135-137; the results in page 13, line 259-263, line 270-271, page 14, line 290-292; and the data in supplementary Fig. 1.

Comment 3: The article would benefit for re-organization of the results, discussion and abstract. Normally, the stepwise scheme would start by the in vitro analysis of the anti-infllamatory effect of UC-MSC in the macrophage cell line, and once proved, jumping into the in vivo analysis of UC-MSC in an animal model of ALF. In addition, the discussion and abstract should be organized with the same scheme.

Reply 3: Thank you for the suggestion. Our current study is designed to evaluate the therapeutic effects of UC-MSCs on acute liver injury, and explore the underlying mechanisms. However, accumulating evidence revealed that UC-MSCs have multiple biological functions through multiple targets in various cell types. In the present study, we focused on the role of UC-MSCs on macrophage, an important innate immune cell type in acute liver injury. We would be pleased to re-organize the scheme as you suggested, if still needed.

Changes in the text: none.

Comment 4: In line 129, the nomenclature of the groups is established. However, in the results section, the authors used “model group” or “ALF mice” without sticking to the nomenclature in methods. In addition, the group “control+UC-MSCs” would be better named as “UC-MSC” alone. The authors must keep consistency in the way they name the groups of mice throughout the manuscript. Please, change accordingly.

Reply 4: As suggested, we revised the “model group” and “ALF mice” to “D-GalN/LPS group”. The group “control+UC-MSCs” was changed to “UC-MSCs” alone.

Changes in the text: Please see page 7, line 126; page 12, line 245, 247, 250; page 13, line 268; page 14, line 277, 286; page 15, line 301; page 25, line526.

Comment 5: The authors injected UC-MSC to the mice, after culturing them in vitro. However, the authors have not determined the cell viability of the culture cells before injection. From the total number of cells injected, the percentage of viable cells that are inducing the anti-inflammatory effect should be calculated and clearly stated.

Reply 5: Thanks for the suggestion. We tested the cell viability and added this information in the revised manuscript.

Changes in the text: Please see page 12, line 241.

Comment 6: Line 312, the authors discuss that hepatocellular necrosis and inflammation are the main features of liver failure. However, in line 334 the authors said that hepatocyte apoptosis is the crucial contributor of acute liver disease. This inconsistency should be addressed as necrosis and apoptosis are completely different mechanism of cell death. Indeed, acute liver injury is characterized by cellular necrosis, which leads to regeneration of the tissue by the uninjured hepatocytes. In the manuscript, the authors only focused on cellular apoptosis, without analyzing the level of necrosis within the animal model used.

Reply 6: As suggested, we have revised the description about the hepatocyte apoptosis to make the expression consistent. We also have quantified the area of necrosis of H&E staining images in Figure 1.

Changes in the text: Please see page 16-17, line 342-343 and the quantification of necrosis of H&E staining in Fig 1F.

Comment 7: In line 342, the authors say "UC-MSCs markedly attenuated hepatocyte apoptosis very likely through inhibiting TNFα production". The authors should avoid overconclusions about the anti-inflammatory mechanism of UC-MSC without clear proof.

Reply 7: Thanks for the suggestion, we have modified the description.

Changes in the text: Please see page 17, line 349-350.

Comment 8:

Figures: the authors should improve the quality of all figures.

Figure 1: The units of plasma ALT and AST must be corrected (it is μL, instead of UL). Subsection C include also control livers; please, change the legend accordingly. Legend of E says "serum" but in the graph says "plasma". H&E must be written without abbreviation.

Figure 2 does not show the loading control of the western blot. The histological pictures must be bigger, and the name of each group outside the picture. The meaning of the arrow heads is not given. The graph of tunnel assay does not include the values of the control groups. How many histological fields were chosen for quantification of apoptotic cells?

Figure 3: the authors do not mention the meaning of the increased DAPI staining in D-GalN/LPS groups with CD3. Is that an increase in lymphocyte infiltration in that area?

Figure 4: the legend does not match with the subfigures. Where's D?

Reply 8:

Thank you for the suggestion about the images' quality. We have improved the quality of all figures.

Figure 1: We checked the graph and found your doubt may be due to the blurry picture. We used U/L as the units according to the manufacturer's instruction. The legend of Figure 1C changed accordingly, including the control livers. The graph of Figure 1E changed to "serum". H&E was written in full name.

Figure 2: We have added the loading control of the western blot. We corrected the tunnel histological pictures and the name. The meaning of the arrow heads were described in the legend. The values of the control groups have been incorporated in the graph of tunnel. The numbers of histological fields were given in the legend.

Figure 3: Increased CD3 staining meant an increase in lymphocyte infiltration which has been mentioned in the section of Results.

Figure 4: We are sorry for the mistake. "D" has been deleted.

Changes in the text:

Please see Fig 1E, and page 25, line 528;

Fig 2A, and page 25, line 534;

Page 14, line 287-290.

Comment 9: Minor aspects:

Introduction

Line 74: NLRP3 induces also the maturation of IL-18. The authors should mention it in the introduction, and justify why this cytokine was not assessed too.

Reply 9: Thanks for your suggestion. We have mentioned the maturation of IL-18 in the section of Introduction. Besides, we added the concentration of IL-18 in the liver.

Changes in the text: Please see page 4, line 73, and the Fig 4C.

Comment 10: Line 79: substitute "is" by "was" in the text "…for is identified…"

Reply 10: We have revised it.

Changes in the text: Please see page 4, line 77.

Comment 11: the authors should clarify the superiority of UC-MSC compared to MSC. Do UC-MSCs keep self-renewing ability and multiple differentiation potential?

Reply 11: We have clarified the superiority of UC-MSC compared to MSC in the section of Introduction.

Changes in the text: Please see page 5, line 85-87.

Comment 12: Methods

Line 109: what kind of serum-free medium from Lonza was used to culture UC-MSCs?

Reply 12: We have added the type of serum-free medium in the section of Methods.

Changes in the text: Please see page 6, line 107.

Comment 13:

Line 111: the authors should explain why they collected UC-MSCs after five generations of culture.

Reply 13: According to our previous studies, the fifth generation had better purity, rapid proliferative ability, and powerful immunomodulatory function.

Changes in the text: Please see the citation (19) in page 6, line 112.

Comment 14:

Line 115: substitute “were” by “was” when referring to the supernatant.

Reply 14: We have revised it.

Changes in the text: Please see page 6, line 113.

Comment 15:

Line 125: add “animals” after “Laboratory”.

Reply 15: Done

Changes in the text: Please see page 7, line 123.

Comment 16:

Line 145: the primers used for GAPDH must be also specified.

Reply 16: We have added the primer of GAPDH.

Changes in the text: Please see page 8, line 164-166.

Comment 17: Results

Line 244: use past tense (“observed” instead of “observe”)

Reply 17: We have changed the tense.

Changes in the text: Please see page 12, line 246.

Comment 18:

Line 246: “improved” must be substitute by “prevented”

Reply 18: Done

Changes in the text: Please see page 12, line 248.

Comment 19:

Line 255-256: the sentence should be rewritten for better understanding.

Reply 19: The sentence has been rewritten.

Changes in the text: Please see page 13, line 257-258.

Comment 20:

Line 267: was the ratio of Bcl-xl/Bax significantly different from controls? In addition, the authors should implement a short introduction of caspase 3 as a pro-apoptotic molecule.

Reply 20: We have added the description of the ratio of Bcl-xl/Bax compared with controls. A short introduction of caspase 3 was also added.

Changes in the text: Please see page 13, line 274, 276.

Comment 21:

Line 287: the role of NLRP3 inflammasome on ALF should include at least one reference study.

Reply 22: Reference has been added.

Changes in the text: Please see page 14, line 298.

Comment 23:

Line 294: UC-MSC affected NLRP3 inflammation sounds weird. Please, change the verb, for example "reduced".

Reply 23: "Affected" has been replaced by "reduced".

Changes in the text: Please see page 15, line 304.

Comment 24:

Discussion

Line 324: the sentence lacks sense. Please, rewrite.

Reply 24: We have rewritten the sentence.

Changes in the text: Please see page 16, line 331-332.

Comment 25: must read "transplantation".

Reply 25: Done

Changes in the text: Please see page 16, line 332.

Comment 26: Line 327 and 330: remove the number of cells administered. These data must be shown in results, not in discussion.

Reply 26: The number has been removed.

Changes in the text: Please see page 16, line 334.

Comment 27:

Line 345: the authors say “previous studies” but only place one reference. Please, correct accordingly.

Reply 27: We have revised it.

Changes in the text: Please see page 17, line 353.