Dear Editor and Reviewers,

Thank you for your letter and the reviewer's comments concerning our manuscript (NO.: 85722). Those comments are valuable and very helpful. We have read the comments carefully and have made corrections. Revisions in the manuscript are shown using the yellow highlight for additions. We have standardized the use of abbreviations, uploaded PPT files of editable figures 1-9, a completed conflict-of-interest statement, and a copyright license agreement with all authors' signatures. Also, we have shown the statistical analysis results of the wet-to-dry weight ratio, the cell mycoplasma detection report, the immunofluorescence identification report, and a graphical abstract according to the reviewer's comments. In addition, the revised manuscript has been polished by a professional organization and relevant certificates have also been uploaded. All changes in the manuscript have been marked with a yellow background. Hope to meet the requirements. The responses one-by-one to the reviewer's comments are presented and comments are numbered, and the responses are red.

Reviewer #1:

1. In the abstract section, the number of mice was written as "C57BL/6 mice were randomly divided into four groups (each group has 12 rats) including the sham, sham + MSC, LPS, and LPS + MSC groups, with 18 mice in each group". So, the number of mice is 12 in each group or 18 in each group??

Response: Thank you for your reminder. Each group consists of 18 mice, and we have corrected this error. (Page 3)

2. The abstract needs to be summarized and no need to add the results in detail (mean and standard deviation).

Response: We have removed the detailed content of the results from the abstract and have streamlined the abstract. (Page 4)

3. In materials and methods: “The three mice in each group were randomly taken and weighed, then, lung was baked in an oven at 80°C for 48 h …….”, the number of mice is three. Based on previous information about the number of mice in each group, the reviewer asks for the SPSS of the statistical results.

Response: Thank you for your careful review and professional comments. To comply with the principle of “Reduction, Replacement, Refinement”, we tested the ratio of lung wet/dry weight with three mice in each group as Li et al. reported [1]. To minimize experimental error, each mouse's left and right lungs were evenly divided into two parts, in other words, each mouse's lungs were evenly divided into four parts for the experiment. Finally, data were analyzed by SPSS. We have shown the SPSS analysis results in Figure 1 below. In addition, we have added more specific experimental operation descriptions in the experimental methods.

**Multiple Comparisons**

Dependent Variable: Wet_to_Dry

| | (I) 分组 | (J) 分组 | Mean Difference (I-J) | Std. Error | Sig. | 95% Confidence Interval | |
|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound |
| Tukey HSD | Sham | Sham+HUC-MSCs | .27000 | .72096 | .982 | -1.6550 | 2.1950 |
| | | LPS | -2.55583* | .72096 | .005 | -4.4808 | -.6309 |
| | | LPS+HUC-MSCs组 | -.54833 | .72096 | .872 | -2.4733 | 1.3766 |
| | Sham+HUC-MSCs | Sham | -.27000 | .72096 | .982 | -2.1950 | 1.6550 |
| | | LPS | -2.82583* | .72096 | .002 | -4.7508 | -.9009 |
| | | LPS+HUC-MSCs组 | -.81833 | .72096 | .670 | -2.7433 | 1.1066 |
| | LPS | Sham | 2.55583* | .72096 | .005 | .6309 | 4.4808 |
| | | Sham+HUC-MSCs | 2.82583* | .72096 | .002 | .9009 | 4.7508 |
| | | LPS+HUC-MSCs组 | 2.00750* | .72096 | .038 | .0825 | 3.9325 |
| | LPS+HUC-MSCs组 | Sham | .54833 | .72096 | .872 | -1.3766 | 2.4733 |
| | | Sham+HUC-MSCs | .81833 | .72096 | .670 | -1.1066 | 2.7433 |
| | | LPS | -2.00750* | .72096 | .038 | -3.9325 | -.0825 |
| LSD | Sham | Sham+HUC-MSCs | .27000 | .72096 | .710 | -1.1830 | 1.7230 |
| | | LPS | -2.55583* | .72096 | .001 | -4.0088 | -1.1028 |
| | | LPS+HUC-MSCs组 | -.54833 | .72096 | .451 | -2.0013 | .9047 |
| | Sham+HUC-MSCs | Sham | -.27000 | .72096 | .710 | -1.7230 | 1.1830 |
| | | LPS | -2.82583* | .72096 | .000 | -4.2788 | -1.3728 |
| | | LPS+HUC-MSCs组 | -.81833 | .72096 | .262 | -2.2713 | .6347 |
| | LPS | Sham | 2.55583* | .72096 | .001 | 1.1028 | 4.0088 |
| | | Sham+HUC-MSCs | 2.82583* | .72096 | .000 | 1.3728 | 4.2788 |
| | | LPS+HUC-MSCs组 | 2.00750* | .72096 | .008 | .5545 | 3.4605 |
| | LPS+HUC-MSCs组 | Sham | .54833 | .72096 | .451 | -.9047 | 2.0013 |
| | | Sham+HUC-MSCs | .81833 | .72096 | .262 | -.6347 | 2.2713 |
| | | LPS | -2.00750* | .72096 | .008 | -3.4605 | -.5545 |

*. The mean difference is significant at the 0.05 level.

Figure 1 The SPSS analysis results of the ratio of wet to dry lung weight.

4. Provide the catalog number, source, and the name of the country for tumor necrosis factor (TNF)-α, IL-1β, and IL-6 ELISA kits.

Response: Thank you for your reminder. We have added the catalog number, source, and country of ELISA kits. (Pages 9-10)

5. What was the rationale for using HUC-MSCs specifically?

Mesenchymal stem cells have been shown to have a beneficial effect on ALI [2]. Currently, two types of MSCs, bone marrow-MSCs (BM-MSCs) and Human umbilical cord MSCs (HUC-MSCs) are most used in clinical and scientific research. However, bone marrow mesenchymal stem cells (BM-MSCs) come from limited sources, have to be obtained through invasive

surgery, and their proliferative capacity decreases with donor age [3]. HUC-MSCs have become an attractive candidate for stem cell research and applications because of their specific advantages, including easily obtained, abundant source, and no tumorigenicity, especially no ethical controversy [4]. Although scientists have found that umbilical cord mesenchymal cells can improve ALI, the mechanism of their action is still unclear [5]. Therefore, this study investigated the potential mechanism of action of umbilical cord mesenchymal cells in improving ALI.

6. The introduction and discussion need to be summarized.

Response: According to this comment, we have streamlined the introduction and discussion, summarized the main idea of each paragraph, and adjusted the logical structure to make it easier to understand.

7. The manuscript Needs extensive language editing.

Response: Thank you for your suggestion. The revised manuscript has been edited by a professional language polishing agency. The modified certificate has been uploaded as an attachment.

Reviewer #2:

**1.** Very important information about mesenchyme stem cells.

Response: The purity of cells identified by immunofluorescence is over 90%, and the Mycoplasma detection report shows that Cells were negative for Mycoplasma contamination. The results of the Mycoplasma Test Report and Immunofluorescence Identification Report are shown in the following Figure 2. In the revised manuscript, we supplemented "The mycoplasma free HUC-MSCs (HUM-iCell-e009) were purchased from iCell Bioscience Inc. (Shanghai, China) and cultured in a specialized medium (PriMed-iCELL-012, iCell Bioscience, China) containing supplements at 37°C in a 5% $CO_2$ incubator. Purity of HUC-MSCs was assessed by immunofluorescence and was typically

greater than 90%. Cell identification conducted by iCell Bioscience Inc." (Page 8)

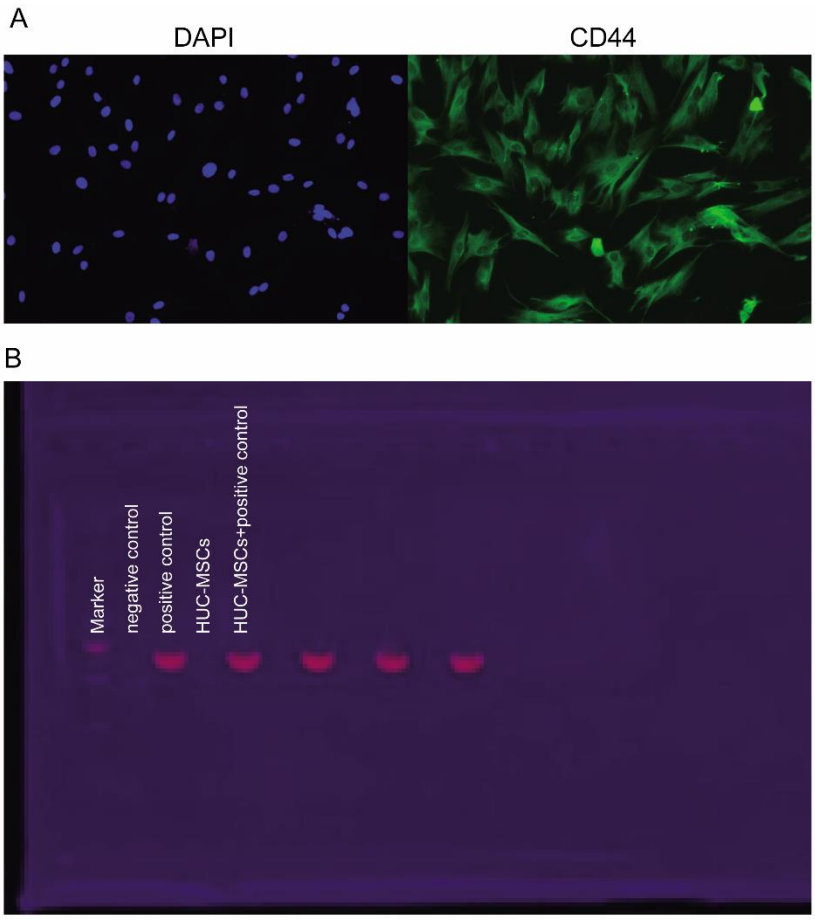


Figure 2 Results of immunofluorescence identification and the mycoplasma test. A: DAPI and CD44 immunofluorescence imaging of HUC-MSCs (×200). B: Mycoplasma detected by PCR. Cell identification conducted by iCell Bioscience Inc.

**Reviewer #3:**

1. Title: The title is not appropriate. I suggest making up it.

Response: Thank you for your professional suggestion. We have revised the title to "Correlation between gut and lung microbiota homeostases and human umbilical cord mesenchymal stem cells in acute lung injury". Looking forward to meeting your requirements. (Page 1)

2. Paper is replete with some grammatical mistakes. Needs rewriting and thorough evaluation. For example, “that HUC-MSCs improve ALI by via lung-gut microflora”.

Response: We checked the whole manuscript, and corrected spelling and English Syntax errors. Additionally, the revised manuscript has been edited by a professional language polishing agency. The modified certificate has been uploaded as an attachment.

3. Some references missing. For example, " The homeostasis of gut microbiota is reported to be important for human health including modulatory effects on acute lung injury.” etc.

Response: According to this comment, we have supplemented some references in the introduction and method part.

4. In order to make the paper more interesting to read, I suggested that the authors could add one graphical abstract to the manuscript.

Response: Thank you for your suggestion. We have added a graphical abstract.

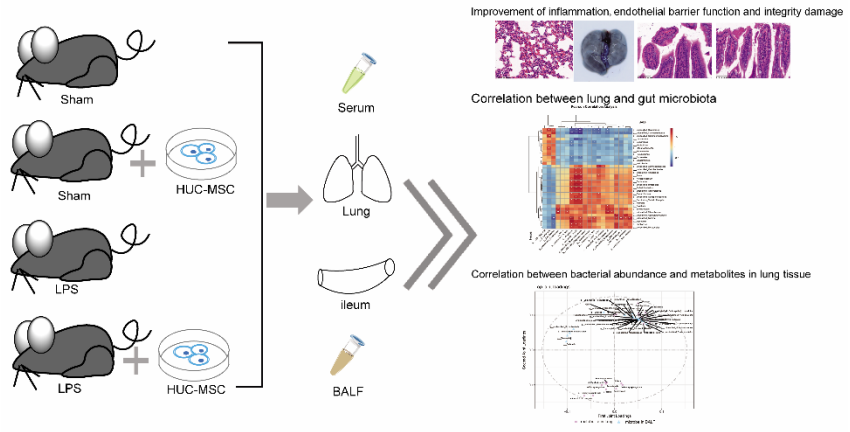


Figure 3 The graphical abstract. Correlation between gut and lung microbiota homeostases and human umbilical cord mesenchymal stem cells in acute lung injury.

5. I suggest including clear limitations of the study in the discussion.

Response: We supplemented “Naturally, this study only examined the correlation between microarray and metabolomics in the lung and gut and

does not have conclusive evidence to confirm that the lung-gut axis microbiota is a crucial factor behind the ability of HUC-MSCs to enhance ALI. Animal and clinical studies are necessary to validate the role of gut and lung microorganisms in the cellular improvement of HUC-MSCs in ALI" in the revised manuscript. (Pages 21-22)

Finally, we hope that the manuscript will meet the requirements for publication. Thank you again.

Sincerely,

En-Hai Cui

References


1. Li, Y., J. Xu, W. Shi, C. Chen, Y. Shao, L. Zhu, W. Lu, and X. Han, *Mesenchymal stromal cell treatment prevents H9N2 avian influenza virus-induced acute lung injury in mice.* Stem Cell Res Ther, 2016. **7**(1): p. 159.
2. Xu, Y., J. Zhu, B. Feng, F. Lin, J. Zhou, J. Liu, X. Shi, X. Lu, Q. Pan, J. Yu, Y. Zhang, L. Li, and H. Cao, *Immunosuppressive effect of mesenchymal stem cells on lung and gut CD8(+) T cells in lipopolysaccharide-induced acute lung injury in mice.* Cell Prolif, 2021. **54**(5): p. e13028.
3. Baker, N., L.B. Boyette, and R.S. Tuan, *Characterization of bone marrow-derived mesenchymal stem cells in aging.* Bone, 2015. **70**: p. 37-47.
4. Kakabadze, Z., N. Kipshidze, T. Paresishvili, N. Kipshidze, Z. Vadachkoria, and D. Chakhunashvili, *Human Placental Mesenchymal Stem Cells for the Treatment of ARDS in Rat.* Stem Cells Int, 2022. **2022**: p. 8418509.
5. Tu, C., Z. Wang, E. Xiang, Q. Zhang, Y. Zhang, P. Wu, C. Li, and D. Wu, *Human Umbilical Cord Mesenchymal Stem Cells Promote Macrophage PD-L1 Expression and Attenuate Acute Lung Injury in Mice.* Curr Stem Cell Res Ther, 2022. **17**(6): p. 564-575.

Chief Editor Specific comments:

1) The current version of the title is neither logical nor captured about the data sets, as Reviewer 3 pointed out.

Response: We changed the title to "Unraveling improvement effect of human umbilical cord mesenchymal stem cells on acute lung injury via regulating the lung-gut axis - integrated 16S rDNA sequencing and non-targeted metabolomics analyses". (Page 1)

2) Page 38: "Figure 3 Human umbilical cord mesenchymal stem cells improve histopathology, inflammation, and endothelial barrier integrity of the ileum in acute lung injury mice." Why did they use human UC MSCs in mouse models without using any immune suppression? What was the immune profiling they assessed?

Response: Human umbilical cord mesenchymal stem cells (hUC-MSCs) have been shown to have low immunogenicity, which allows them to be used in acute lung injury mouse models without the need for immune suppression (Lee et al. 2014). The immune profiling typically involves assessing the expression levels of various cytokines, chemokines, and growth factors. Given the proven immune regulatory capacity of human umbilical cord stem cells in ALI mice (Wu et al. 2022), this manuscript evaluates a particular set of immune cells and inflammatory cytokines associated with ALI to verify the immunomodulatory effects of hUC-MSCs through the use of Wright's staining and ELISA in this manuscript.

3) Pages 39-40: Figure 4, all panels A - F, the images and text are blurry. A 1200 dpi resolution should be used.

Response: We have provided an editable PPT drawing file for Figure 4. Additionally, as we are unsure whether the PPT file has an impact on pixels, we have also provided an editable AI format of Figure 4. It is worth noting

that we have also modified Figure 4 in the manuscript, however, we are not sure if the image formats used are correct and need further confirmation from the Editors.

4) Page 54, Fig 10: The right half of the images are blurry, and A 1200 dpi resolution should be used.

Response: We have provided an editable PPT drawing file for Figure 10. In addition, we have rearranged the images to increase clarity. As we are unsure whether the PPT file has an impact on pixels, we have also provided an editable AI format of Figure 10. It is worth noting that we have also modified Figure 10 in the manuscript, but we are not sure if the image formats used are correct. There are need further confirmation from the Editors.

5) Page 7: "The purity of HUC-MSCs was assessed by immunofluorescence and was typically greater than 90%." – How could they evaluate hHU-MSC by immunofluorescence? They need to follow the international standards of MSCs panel biomarkers. What is their definition of hHU-MSCs? Fig 2 is not sufficient.

Response: We have supplemented the flow cytometry identification results of HUC-MSCs according to the criteria of the International Society for Cellular Therapy (Dominici et al. 2006) and some references (Davies et al. 2019; Wu et al. 2022).

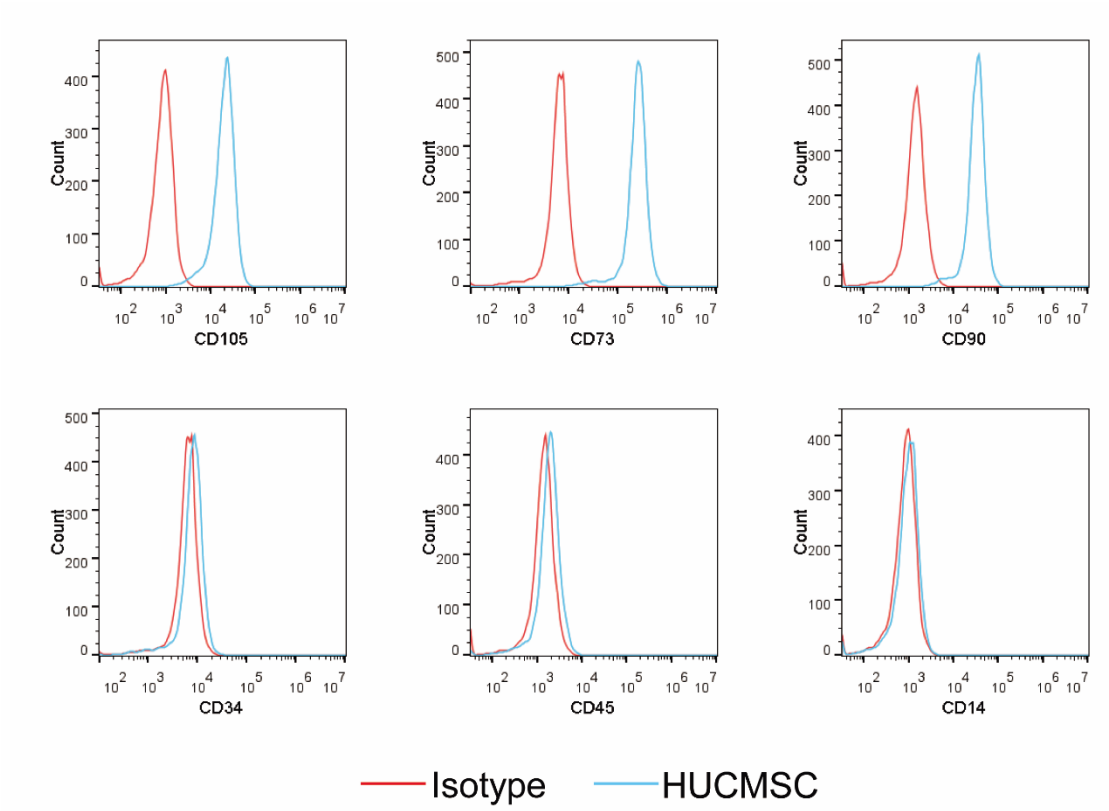


Figure 1 The flow cytometry identification results of HUC-MSCs. HUC-MSCs ($1\times10^5$ cells) were incubated with antibodies in the dark for 30 min and flow cytometry were performed by a Novocyte flow cytometer (Agilent, CA, USA).

6) Page 7: "A total of 48 6-8-wk-old male 7BL/6 mice were purchased from Beijing." Page 8:" The random number method was used to divide mice into four groups, namely, sham, sham + MSCs, LPS, and LPS + MSCs groups, with 18 mice in each group. The 36 randomly selected mice were intraperitoneally injected with 100 mL of LPS (10 mg/kg) to induce ALI[7], and sham mice were administered 100 mL of 0.9% NaCl as controls." Reviewer #1: 1. In the abstract section, The number of mice was written as "7BL/6 mice were randomly divided into four groups (each group has 12 rats). Page 3: "METHODS, 7BL/6 mice were randomly divided into four groups (18 rats per group)." Comment: 18X4 = 72 mice. Why did they state a total of 48 mice? 18 rats? , 7BL/6 mice? Why did they state a total of 18 mice per group? Any consideration of statistical power?

Response: We used 18 mice in each group for the experiment, following the "3R" principle. We apologize for the errors in the manuscript and have corrected "48" to "72".

7) English grammar errors crawl across pages, manifested in neither logical nor cohesive. E.g., ". Looking forward to it meeting your requirements. (Page 1) (page 5 of the Rebuttal)

Response: We have corrected it. In addition, we have also briefly corrected the small errors in Word file of "the first round point-by-point responses" without changing the original intention.

8) Page 8: "After 6 h, half of the ALI mice and half of the sham mice were given 0.5 mL of phosphate-buffered saline (PBS) containing HUC-MSCs ($2 \times 10^6$ cells/mL) by intraperitoneal injections[24]" (page 8). This statement contradicts their abstract, which states that "After 6 h, mice were intervened with 0.5 mL phosphate-buffered saline (PBS) containing $1 \times 10^6$ HUC-MSCs by intraperitoneal injection" (page 3).

Response: We apologize for not discovering this error during the previous revision. The description of the abstract is correct, and we have corrected the errors in the manuscript.

9) Reviewer 1, point #3: "In materials and methods: "The three mice in each group were randomly taken." The author's rebuttal was insufficient because they used 18 mice per group.

Response: We have a total of 18 mice in each group, of which 3 were randomly selected for analyzing the wet-to-dry weight ratio of the lungs.

10) “Non-Native Speakers of English Editing Certificate” contains a typo: acute lung injur” – not injur but injury.

Response: We resubmit the certificate.

Furthermore, we extend our sincere appreciation to the editors and reviewers for their meticulous assessment and remarkable efficiency. We are confident that our collaboration will be delightful.

Sincerely,
En-Hai Cui

**References:**

Davies LB, Jones RH, Thornton CA. 2019. Maternal Serum, an Isolation and Expansion Tool for Umbilical Cord Matrix Mesenchymal Stromal Cells. Tissue Eng Part C Methods. 25(4):p. 213-221. eng.

Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D, Horwitz E. 2006. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy. 8(4):p. 315-317. eng.

Lee M, Jeong SY, Ha J, Kim M, Jin HJ, Kwon SJ, Chang JW, Choi SJ, Oh W, Yang YS et al. 2014. Low immunogenicity of allogeneic human umbilical cord blood-derived mesenchymal stem cells in vitro and in vivo. Biochem Biophys Res Commun. 446(4):p. 983-989. eng.

Wu KH, Li JP, Chao WR, Lee YJ, Yang SF, Cheng CC, Chao YH. 2022. Immunomodulation via MyD88-NFκB Signaling Pathway from Human Umbilical Cord-Derived Mesenchymal Stem Cells in Acute Lung Injury. Int J Mol Sci. 23(10):p. eng.