Peer Review File
Article information: https://dx.doi.org/10.21037/jtd-22-1669

Reviewer A

In material and Methods:

1. **Researchers please mention the reference number in section b.FMMNC preparation.**

Reply 1: Thanks for the kind comment. The reference of section b.FMMNC preparation is [16] Wen C, Liu XY, Wan WQ, Yi ZW. Effects of Fetal and Neonatal Murine Peripheral Blood Mononuclear Cells Infusion on MicroRNA-145 Expression in Renal Vascular Smooth Muscle Cells in MRL/lpr Mice. Transplant Proc 2015; 47:2523-7.

Changes in the text: We have modified our text at section **b.FMMNC preparation** as "Fetal mouse blood was harvested from jugular vein of 10 fetal mice as previously described (16) and …" (see Page 6, line 138) and update the section **References**.

2. **Researchers, please explain why asthma is a lung disease, why they used FMMNC in I.V. Is the possible migration of these cells to the lung tissue?**

Reply 2: Thanks for this comment.

(1) As mentioned in Global Strategy for Asthma Management and Prevention (GINA), asthma is a heterogeneous disease characterized by chronic airway inflammation, which is defined by the history of respiratory symptoms such as wheezing, shortness of breath, chest tightness, or cough that vary over time and in intensity, together with variable expiratory airflow limitation. Thus, asthma is mainly a lung disease.

(2) Lung has rich capillary network. When the cell suspension is intravenously injected, most of the cells can reach the lung as a first-line barrier in several minutes (PMID: 19570514, PMID: 19099374) to several days (PMID: 30133167). Thus administrating of cells therapy via intravenous injection is commonly used in experimental animal study on lung diseases such as asthma, COPD, lung fibrosis and ARDS (PMID: 26753925, PMID: 31608724). Although intratrachial instillation is an alternative way in cell therapy for lung diseases, intravenous injection is used more commonly as its better accuracy in treatment dose.

(3) UCMNCs consist of various kinds of cells including MSCs, hematopoietic cells, endothelial progenitor cells, immune cells. Adhesion molecules such as integrins and VCAM-1 on MSCs/endothelial cells, and the mechanical trap of capillary (PMID: 33664277) can mediate the interaction or retention of MSCs with lung tissue. But it seems that the allograft of MNCs can rarely engraft and differentiate into lung tissue (PMID: 31815001).

Changes in the text: We have modified our text as "As most of intravenously injected exogenous cells can be enriched at lung capillary network for up to several days (17), mice in the treatment groups were intravenously injected via tail vein in 30s with 100 μl of cell suspension per mouse with low, medium, or high doses of

MNCs on days 7 and 20, …" (see Page 6, line 152-155) and added PMID: 30133167 as new reference 17.

3. Briefly explain how you practically injected the cells?

Reply 3: Thanks for this comment. 100 μl of cell suspension per mouse with low, medium, or high doses of MNCs was intravenously injected via tail vein in 30s.

Changes in the text: We have modified our text as "As most of intravenously injected exogenous cells can be enriched at lung capillary network for up to several days (17), mice in the treatment groups were intravenously injected via tail vein in 30s with 100 μl of cell suspension per mouse with low, medium, or high doses of MNCs on days 7 and 20, …" (see Page 6, line 152-155) and we have correct the cell concentrations for i.v. injection in section **b. FMMNC preparation** as shown in Page 6-7, line 144-146.

4. Given that different parts of the lung are involved in the asthma of the animal model differently and show a different intensity of tissue involvement, the researchers please write which part of the lung is done to check sampling pathology?

Reply 4: Both left and right lung lobes were embedded together in one paraffin block. And the largest longitudinal section of the lung lobes were stained using hematoxylin and eosin (HE) to semi-score the airway inflammation level. But we didn't discriminate which was the left or right lung lobe on the slide.

Changes in the text: We have modified our text in the section **Histopathological analysis** as "The largest longitudinal section of the lung lobes were stained using hematoxylin and eosin (HE). (see Page 8, line 186)

In Results:

5. In this research, Th1/Th2 immune imbalance is mentioned. But the researchers did not measure the level of Th1 (IFN-gamma) cytokine.

Reply 5: Thanks for this comment. Th1/Th2 imbalance is a classic theory of asthma inflammation mechanism, which is inevitably introduced in most asthma-relative studies and serves as an important theoretical background for research. The concentration of IFN-gamma was not detected in this study, but Th2 related factors IL4, IL5 and IL13 were detected in BALF and serum. All these three cytokines significantly increased in BALF, indicating a significant enhancement of Th2 inflammation, which was a manifestation of imbalance.

6. Does device Sysmex animal hematology analyzer (XN-1000V, Sysmex Corporation, Japan) really have the ability to differentially count mouse cells? Please provide evidence for the accuracy and sensitivity of this count.

Reply 6: Thanks for the comment. Several literature have compared the ability of Sysmex animal hematology analyzer (XN-1000, XT-2000) on differential leukocyte counting, and found that it has good linearity and is comparable to manual counting (PMID: 32519581, PMID: 8729435, DOI:10.1007/s00580-006-0655-x). In manual

counting, usually only 100-400 cells are counted per sample, while automatic counting has a larger number of cells per sample. But we didn't repeat to test the accuracy and sensitivity of this automatic method in our own research.

Changes in the text: We have added the (PMID: 32519581) and (DOI:10.1007/s00580-006-0655-x) as new reference 19 and 20 in section **Analysis of bronchoalveolar lavage fluid (BALF) and serum.** (see Page 8, line 176)

7. **The mice of UCMNCM (medium-dose) group, had the best therapeutic effects in this research. In Figure 3 and in UCMNCH group, mucus can be seen inside the alveoli. Was no mucus secretion seen in any of UCMNCM (medium) group? Have photographs been taken of the identical parts in the lung?**

Reply 7: Thanks for the comment. Mucus hypersecretion is a common symptom of asthma. Actually, mucus secretion could be found in all the groups except normal control group and our interventions (both UCMNC and FMMNC) showed no significant effects on the mucus hypersecretion in the asthmatic mice. the largest longitudinal section of the lung lobes were stained using hematoxylin and eosin (HE) to semi-score the airway inflammation level. however it was still difficult to take photographs at the identical parts in different lungs. To improve comparability, we have modified the Figure 3 with photographs with similar size of airways and was representative to the inflammation situation of the section.

Changes in text: The Figure 3 have been modified to improve comparability.

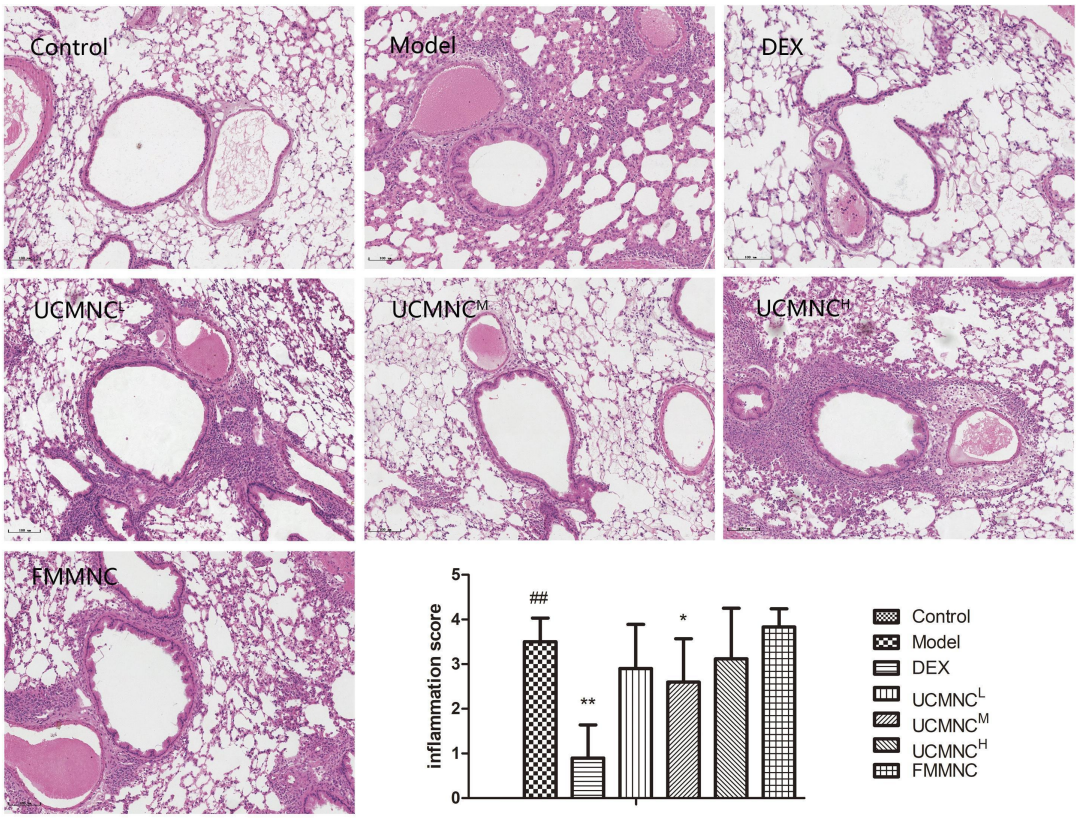

Figure for Reply 7. The revised Figure 3.

8. **Do the researchers have a picture of the stained leukocytes in the BALF fluid?**

Reply 8: Thanks for the comment. In the pre-experiment, we stained the cell pallets in BALF fluid for differential leukocyte counts and took pictures which is shown as follow. However, manual counting is time consuming and prone to errors in cell identification and operator differences. Thus we chose automatic method but did not perform microscopic examination of stained leukocyte in this study.

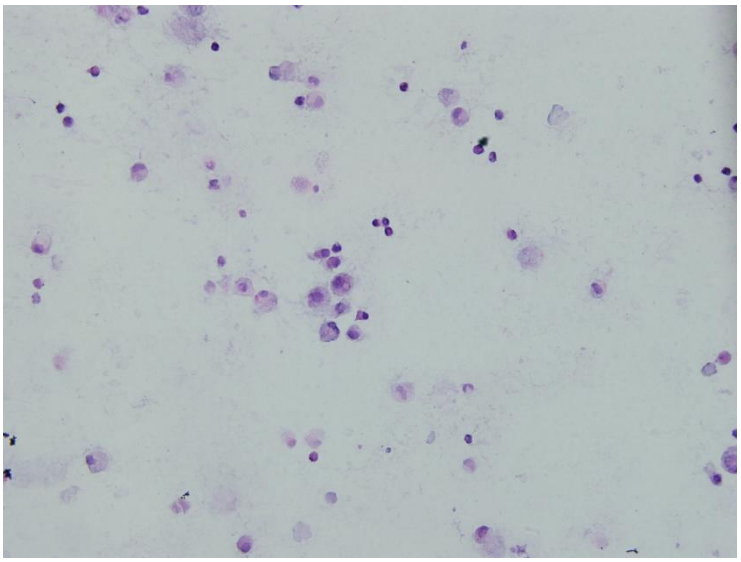

Figure of Reply 8. HE stained leukocytes of BALF from mouse with asthma induced by OVA in pre-experiment (200×).

In Discussion:

9. **Researchers explain from the immunological opinion, how the UCMNCM reduces both the percentage of Eosinophils and Macrophages in BALF?**

Reply 9: Thanks for the comment. According to Figure 2, $UCMNC^{M}$ did not reduce the proportion of macrophages in BALF of asthma mice in this study. A somewhat but not significant decrease of eosinophils was shown in the $UCMNC^{M}$ group and the FMMNC group. UCMNC may reduce the chemotaxis and activation of eosinophils by reducing IL-5 and IL-13, which was discussed in the section **Discussion** (Page 11, line 251-272).

10. **Researchers write about further explanation of mechanism UCMNCM on inflammation and inflammatory cells (an immunological opinion). What mechanism do you suggest for this sentence?**
11. **Our results suggest that human UCMNC administration can reduce asthmatic airway inflammation and injury by inhibiting Th2-related cytokines such as IL-5 and IL-13.**

Reply 10 and 11: Thanks for the comment. It seems that Comment 11 is indicating the "this sentence" in comment 10.

The promoting roles of Th2-related cytokines IL-4, IL-5 and IL-13 in

eosinophilc airway inflammation of asthma have been confirmed in many literature (17). Basing on this, we speculate that the reduced levels of IL-5 and IL-13 in BALF may help to reduce eosinophil infiltration, airway tissue injury, and airway hyperresponsiveness, which were observed in the $UCMNC^{M}$ group in our research. In another hand, besides Th2 cells, IL-5 and IL-13 can be also produced by various cells such as type 2 innate lymphoid cells and mast cells. This study could not find out the target cells of UCMNC in the asthmatic mouse induced by OVA. So we modified the sentence (Comment 11) in revision.

The exact mechanisms of UCMNCs on asthma are not certain yet. UCMNCs consist of hematopoietic stem cells, MSCs and immune cells (5). According a previous study(12) in bone marrow-derived mononuclear cells (BMMNC), CD11b+ immune cells (mainly monocytes, dendritic cells, macrophages) and Sca-1+ MSCs in MNC exert the main beneficial effects on mouse asthma model. Inhibiting the activation of Th2 cells and eosinophils through TGF-beta and IL-10 pathways, and promoting the Treg cells and M2 macrophages to suppress inflammation are considered as important mechanisms for MSC's anti-asthmatic effects, which have been broadly reported in preclinical animal studies (37). The CD11b+ dendritic cells in UCMNC may also induce Treg differentiation and increase M2 polarization, exerting an inhibitory effect on airway inflammation (12).

Changes in the text: We have modified our text in section **Discussion** as "Our results suggest that human UCMNC administration can reduce Th2-related cytokines such as IL-5 and IL-13 and inhibit asthmatic airway inflammation and injury. However, besides the Th2 cells, IL-5 and IL-13 can be also produced by various cells such as type 2 innate lymphoid cells and mast cells. Thus the target immune cells modulated by UCMNC in treating OVA-induced asthma in mouse model are still unclear in present study. "(see Page 11-12, line 266-272)

12. The researchers write about, what the molecular cellular connection is between the UCMNC therapy and the reduction of cell proliferation of CD4+CD8−. While your model has allergic asthma.

Reply 12: As shown in Figure 6, our results showed that high dose of UCMNCs significantly increased the airway CD4+CD8− T-helper cells in asthmatic mice, while FMMNCs significantly increased the CD4−CD8+ T-killer cells and decreased the CD4+CD8− T-helper cells. In allotransplantation and xenotransplantation, the rejection of donor cells by recipient T cells may involve "direct"(donor MHC restricted) or "indirect" (host MHC restricted) antigen recognition. In a study of mouse xenograft heart transplantation, it was found that the rejection response of recipient mice to xenograft rat heart mainly depends on CD4 T cells, and the presentation of donor antigens by the mouse's MHC II is crucial in xenograft rejection (PMID: 22789136). In our study, human UCMNC intravenously injected into mice was a xenograft and also caused a significant increase in CD4 T cells, which may also be related to the presentation of human UCMNC cell antigens by the MHC II of mouse antigen presentation cells. But it should be noted that the mechanism for rejection in one transplant model do not necessarily work for another transplant model.

The details about the rejection of human UCMNC allograft in asthma patients should be accessed in future clinical research.

Changes in the text: We have modified our text in section **Discussion** as “In a study of mouse xenograft heart transplantation, it was found that the rejection of recipient mice to xenograft rat heart mainly depends on CD4 T cells, and the presentation of donor antigens by the mouse's MHC II is crucial in xenograft rejection (36). In present study, human UCMNC intravenously injected into mice was a xenograft and also caused a significant increase in CD4 T cells, which may also be related to the presentation of human UCMNC cell antigens by the MHC II of mouse antigen presentation cells. But it should be noted that the mechanism for rejection in one transplant model do not necessarily work for another transplant model. The details about the rejection of human UCMNC allograft in asthma patients should be accessed in future clinical research.” (see Page 13, line 307-317)

Reviewer B

1. **The experiment design was overly simplistic, consisting of only one mouse experiment with multiple readouts split into multiple figures.**

Reply 1: Thanks for the comment.

MNC is a mixture of various types of immune cells, MSCs and progenitor cells, which is directly isolated from bone marrow, peripheral blood, or umbilical cord blood and does not undergo *in vitro* culture. Cruz FF et al (2016) tested the therapeutic effect of bone marrow-derived MNC on asthma using an *Aspergillus* hyphal extract-induced mouse asthma model, providing important reference for our study and suggesting the potential value of MNC in asthma treatment. The noninvasiveness, high accessibility, and few ethical constraints of UCMNCs make them a suitable alternative to BMMNCs, and UCMNCs have higher cell viability and lower reaction to cytotoxic T cells and Th1 cells (5). So we evaluated the therapeutic effect of UCMNC on asthma using the classic OVA-induced mouse asthma model and examined the indicators commonly used for preclinical evaluation of anti-asthma effect. Glucocorticoid and FMMNC were used as drug control and allogeneic transplantation control. The experiment design can meet the primary objective of this study.

However, we should acknowledge that the design of this study is not complicated enough to conduct in-depth mechanism research. In the future, if resources permit, other asthma animal models (such as house dust mite or toluene diisocyanate-induced mouse asthma models as mentioned in section **Discussion**) can be used to further evaluate the effects of UCMNC on other asthma sub-types. Besides, the intervention protocol of UCMNC might be further optimized in the administration route, frequency, and treatment timing through specially designed experiments.

Changes in the text: We have added the text in section **Discussion** as “Besides, the intervention protocol of UCMNC might be further optimized in the administration route, frequency, and treatment timing through specially designed experiments in future research.”(see Page 14, line 337-339).

2. **The effectiveness of UCMNC at different concentrations does not show a clear dose-response relationship, and the findings presented in different figures are not consistent with each other. The authors have not provided a satisfactory explanation for this.**

Reply 2: Thanks for comment.

MNC is a mixture of various types of immune cells, MSCs and progenitor cells. The study of Cruz FF *et al* (2016) indicated CD11b+ immune cells (mainly monocytes, dendritic cells, macrophages) and Sca-1+ MSCs in bone marrow MNC exert the main beneficial effects on mouse asthma model (12). However, the hematopoietic stem cells in MNC may be enriched in airways after intravenous injection and differentiate into eosinophils under the induction of an allergic inflammatory microenvironment (40). Besides, the possible xenograft rejection in preclinical animal study, and the pulmonary microvascular obstruction caused by high dose of cell injection might also defect the therapeutic effects human UCMNC on experimental asthma in mouse. These pro-and-con effects of UCMNC may result in a not-clear dose-response relationship, which is not rare in studies of cell therapy (14, 30). We have discussed these in the section **Discussion**.

In this study, intravenous injection of medium dose of UCMNCs showed inhibitory effect on IL-5 and IL-13 in BALF of asthmatic mice, while the decreases of IL-5 and IL-13 in serum were slight but not statistically significant. One possible reason is that the injected exogenous UCMNCs tend to be enriched in the capillary network of lung, which may alter the effect degrees of intravenously injected UCMNC on the inflammation cytokines in lung (BALF) and in peripheral blood (serum). However, this down-regulation in cytokines is consistent with the suppression of airway inflammation revealed by histology and the eosinophil inflammation in cytology analysis in our study.

Changes in the text: We have modified the text in section **Discussion** as " One possible reason may be that the injected exogenous UCMNCs tend to be enriched in the capillary network of lung, which may alter the effect degrees of intravenously injected UCMNC on the inflammation cytokines in lung (BALF) and in peripheral blood (serum). However, this down-regulation in cytokines is consistent with the suppression of airway inflammation revealed by histology and the eosinophil inflammation in cytology analysis in this study. "(see Page 11, line 261-266).

3. **As the author mentioned, UCMNC includes UCMSC. Previous studies have shown the effects of UCMSC on asthma (PMID: 28294434), suggesting that they may affect the function of Tregs. It may be less meaningful to using UCMNC with a more diverse cell types.**

Reply 3: Thanks for this comment.

As reported by Cruz FF *et al* (2016), MSC and MNC from mouse bone marrow produced comparable inhibitory effects on the airway hyperresponsiveness, leukocyte infiltration and cytokine levels in asthma mouse model(12). However, UCMNCs can be easily separated from umbilical cord blood by density gradient centrifugation and

used in clinical routines without further *in vitro* manipulation. The *in vitro* manipulation of UCMSC for clinical use usually needs a high-cost certified cell factory and complicated quality control system to avoid any safety risks. Thus UCMNC is also an economical and practical choice.

4. **It is described that there are 20 animals in each group in methodology, but the number of animals in different groups is inconsistent in result part.**

Reply 4: Thanks for the comment.

At the beginning of the experiment, 20 mice were randomly enrolled into each group. And in order to avoid interference of bronchoalveolar lavage operation on histological detection of lung tissue, the 20 mice in each group were divided into two parts: 10 mice were only used for non-invasive airway responsiveness test (the within-group variations is usually higher than invasive RC method, so 10 mice were arranged for this part), and lung tissue was dissected and collected for histology analysis after the provocation test. The remaining 10 mice were used for collecting BALF and blood without airway reactivity testing. A small amount of cell precipitates were taken from each BALF sample for automatic leucocyte counting. Due to limited research funding, flow cytometry analysis of CD4/CD8 T cells and cytokine detection were only performed on the remaining BALF of 5 animals in each group. Some animals died during modeling or tail vein injection operations, resulting in differences in the number of animals. However, the data from all animals that successfully underwent relevant testing were all included in the statistics without "selecting" data.

Changes in the text: We have add a notice in section **Animals** as "Some animals died during modeling or tail vein injection operations, resulting in differences in the number of animals in different groups at the end of experiment."(see Page 6, line 121-122)

5. **The basis for selecting the three concentrations of UCMNC has not been mentioned.**

Reply 4: Thanks for this comment.

The cell concentration used in the study was selected based on preliminary experiments and literatures. We found in preliminary experiment that asthma mice can tolerate the i.v injection of 10^6 and 10^7 UCMNCs per mouse, and previous reports (12) show that 10^6 MSCs and BMMNCs cells per mouse had significant anti-asthmatic effects. Besides MSC, there are some other cell types in MNC. So 2×10^6 UCMNCs per mouse was selected as the medium dose in our study, 10^7 UCMNCs per mouse was selected as the high dose, and 4×10^5 UCMNCs per mouse was selected as the low dose according to the dose gradients.

Changes in the text: We have add text in section **Animals** as "The dose gradient of UCMNCs was set based on preliminary experiments and literatures (12)".(see Page 5 line 119-120)

6. **Asthma is a chronic airway disease characterized by airway inflammation,**

and the significance of interleukin in serum is far less than that in BALF.

Reply 6: In present research, the mouse was challenged by intranasal instillation of OVA to induce experimental asthma. These can induce airway inflammation, which may "spill-over" to systemic inflammation (PMID: 20008867). So it seems rational that the significance of interleukin in serum is far less than that in BALF. In this study, intravenous injection of medium dose of UCMNCs showed inhibitory effect on IL-5 and IL-13 in BALF of asthmatic mice, while the decreases of IL-5 and IL-13 in serum were slight but not statistically significant. One possible reason may be that the injected exogenous UCMNCs tend to be enriched in the capillary network of lung, resulting in different effect degrees of intravenously injected UCMNC on the inflammation cytokines in lung (BALF) and in peripheral blood (serum).

Changes in the text: We have modified the text in section **Discussion** as " One possible reason may be that the injected exogenous UCMNCs tend to be enriched in the capillary network of lung, which may alter the effect degrees of intravenously injected UCMNC on the inflammation cytokines in lung (BALF) and in peripheral blood (serum). "(see Page 11, line 261-264).

7. **The size of the airways in HE staining should be consistent to enhance comparability.**

Reply 7: Thanks for this kind comment. We have modified the Figure 3 as advised, as shown below.

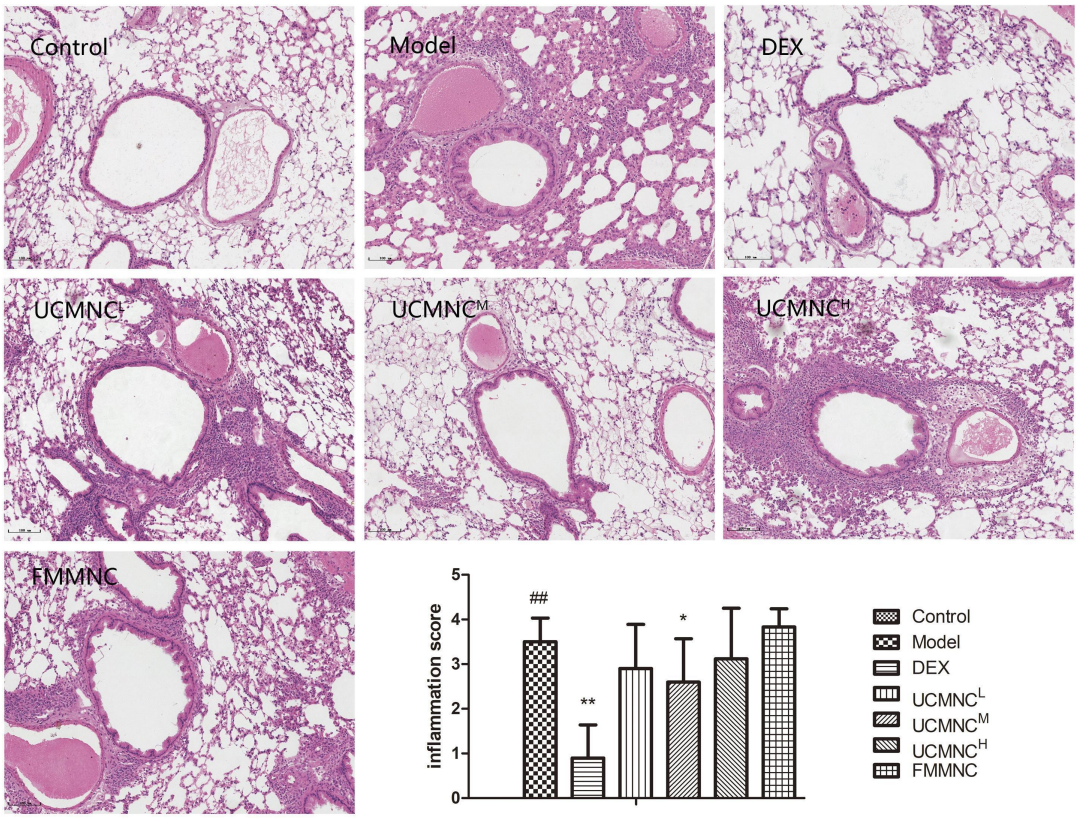

Figure for Reply 8. The revised Figure 3.

8. **The plotting is not standardized, and the airway resistance should be presented as a line chart.**

Reply 8: The airway responsiveness of the mice was assessed using Buxco small animal whole-body plethysmography (WBP) (DSI, USA). It should be noted that the within-group variation of Penh may be higher compared to that of lung resistance and compliance due to the mice being in a conscious state and having more sniffing movements during WBP measurements.

In response to this comment, we attempted to use a line chart to present the results, as shown below. However, the error bars appeared to stack on top of each other, making it difficult to accurately label the statistical significance markers (*) and (#) for each group's line. This may have caused confusion for readers. Therefore, we chose to use a bar chart to display the results in our first draft. **However, any further suggestions from the reviewers and editors regarding the most appropriate form of chart would be greatly appreciated.**

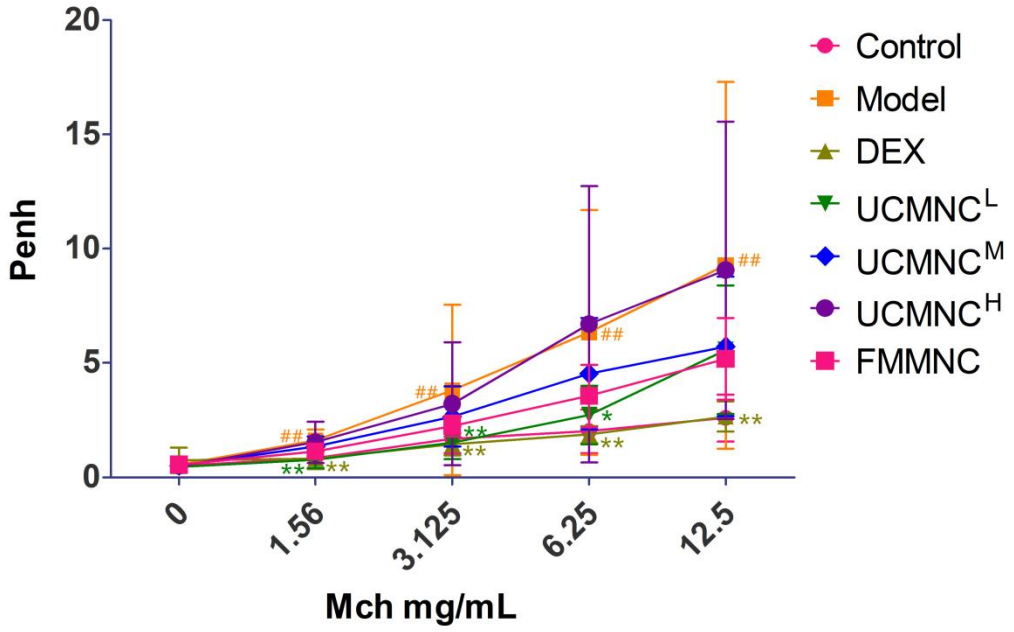

Figure for Reply 8. Line chart of airway responsiveness with SD error bar. However, the error bars appeared to stack on top of each other, making it difficult to accurately label the statistical significance markers (*) and (#) for each group's line. This may have caused confusion for readers.

9. **The experimental method of flow cytometry was poorly described, with no details provided. Lymphocyte subtype analysis in figure 6 should include a description of the flow cytometry strategy and a representative figure.**

Reply 9: Thanks for the comment. We have revised the Figure 6 as advised and added the description of the flow cytometry in section **Methods** and **Figure caption.**

Changes in text: We have modified the text in section **Analysis of bronchoalveolar lavage fluid (BALF) and serum** as "The cell pallets from the BALF were

re-suspended with 100 μL of cold HBSS with 5% fetal bovine serum, and incubated with antibodies anti-mouse CD45-APC-Cy7, anti-mouse CD3-FITC, anti-mouse CD4-APC, and anti-mouse CD8-Percp-Cy5.5 (BD, USA) at the manufacturer's recommended concentration at 4℃ for 30min. Then the cells were washed with FACS buffer and assayed by flow cytometry (FACS Canto2, BD, USA) to measure CD4 and CD8 T cell subsets." (see Page 8 line 176-182) and in section **Figure caption-Figure 6** as " (A) CD4/CD8 lymphocyte cells were gating from CD3+CD45+ cells using flow cytometry. (B) Effect of UCMNCs on CD4/CD8 lymphocyte subsets in BALF." (see Page 21)

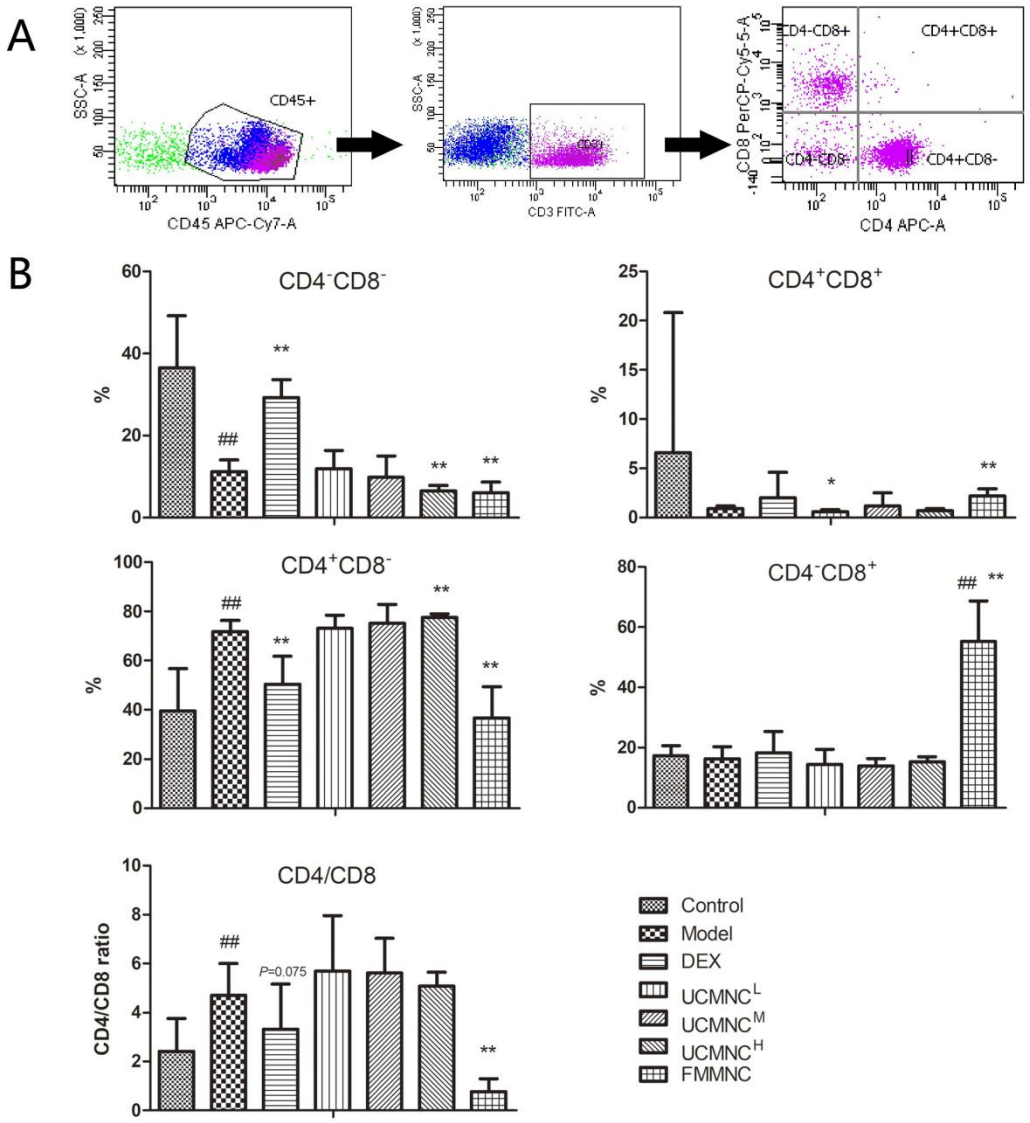

Figure for Reply 9. The revised Figure 6.