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Mesenchymal stem cells alleviate airway inflammation via modulation of T-helper 17/regulatory T cells balance in mice with ovalbumin-induced asthma

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Asthma is considered dysregulated immunity featuring of chronic airway inflammation and remodeling. Mounting evidence reveals that asthmatics show various inflammatory profiles and cell types. Recently, regulatory T cells (Tregs) and T-helper (Th) 17 cells have been described as two distinct subsets from Th1 and Th2 cells and Treg/Th17 imbalance is correlated with the development of asthma.

Mesenchymal stem cells (MSCs), which possess an array of immunosuppressive capabilities are potent modulators of immune responses. Mounting evidence indicates immunosuppressive properties of MSCs in many diseases including asthma.[1,2] However, the mechanisms by which MSCs exert immunomodulatory effects in asthma remain undefined. A recent study demonstrated that MSCs can inhibit Th17 cell differentiation and increase the proportion of forkhead box P3 (Foxp3)$^+$ Treg cells.[3] Herein, we sought to investigate whether MSCs exert anti-inflammatory effects by regulating the Treg/Th17 balance in ovalbumin (OVA)-sensitized mice.

Female BALB/c mice ($n = 8$ for each group; 16–24 g, 6–8 weeks) were provided by the Laboratory Animal Center of Wuhan University (Wuhan, China). All animal experiments were performed following the protocol (No. 2018-159) approved by the Animal Care Committee of Wuhan University (Wuhan, China).

Bone marrow was harvested from femurs and tibias of BALB/c mice, and MSCs were isolated and cultured according to the previous study.[1] Murine asthma model was established as previously reported.[1] BALB/c mice were divided into three groups: (1) control group: mice were sensitized with phosphate buffer saline (PBS), challenged with OVA and treated with PBS; (2) asthma model group (OVA group): mice were sensitized and challenged with OVA and treated with PBS; (3) MSCs treatment group (OVA + MSCs group): mice were sensitized and challenged with OVA and treated with 10^6 MSCs which were injected via tail vein. Twenty-four hours after the last challenge, measurements of airway hyperresponsiveness (AHR) were performed, mice were anesthetized and their tracheas were cannulated via tracheostomy. Increasing concentrations of nebulized methacholine were administered by an ultrasonic nebulizer. AHR was expressed as an enhanced pause (Penh), reflecting pulmonary resistance. Mice were sacrificed using an overdose of 50 mg/kg of pentobarbital 24 h after the last challenge and bronchoalveolar lavage fluid (BALF) was collected, BALF cells were prepared by cytospinning and were stained by Diff-Quick. The numbers of eosinophils, macrophages, neutrophils and lymphocytes were scored by light microscopy. BALF cell pellets were normalized in unit volumes. After BALF samples were obtained, lung tissues were fixed in neutral buffered formalin. The lung tissue was embedded in paraffin, and then stained with hematoxylin and eosin (H&E) solution and periodic acid-Schiff (PAS) to examine the cells that had infiltrated into the peribronchial connective tissues. Goblet cell hyperplasia was reflected by the ratio of PAS-stained cells. Lung inflammation was assessed by a five-point scoring system as previously described[1]: 0, no cells; 1, a few cells; 2, a ring of cells 1 cell layer deep; 3, a ring of cells 2–4 cell layers deep; and 4, a ring of cells more than 4 cell layers deep. The histological mucus index (the percentage of the mucus-positive area of the whole bronchial epithelium) was used to determine the mucus secretion. Levels of

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interleukin (IL)-6, IL-10, IL-17A, and transforming growth factor β1 (TGF-β1) in BALF were measured by enzyme-linked immunosorbent assay according to the manufacturer's instructions.

The percentages of Th17 cells and Treg cells in spleen lymphocytes were measured by flow cytometry. Spleens were removed and lymphocytes were isolated. Cells were distributed into tubes and washed. For Th17 analysis, cells were incubated with allophycocyanin anti-mouse CD4. For Treg analysis, cells were incubated with fluorescein isothiocyanate anti-mouse CD4 and phycoerythrin (PE) anti-mouse CD25. Following surface staining, cells were fixed and permeabilized according to the manufacturer's instructions, and then stained with PE anti-mouse IL-17A for Th17 detection or with APC anti-mouse Foxp3 (eBioscience, San Diego, CA, USA) for Treg detection.

Statistical analyses were performed with GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA). All continuous data with normal distribution were presented as mean ± standard deviation. One-way analysis of variance followed by Dunnett multiple comparison test was used. $P < 0.05$ was considered to be statistically significant.

In our study, MSCs administration markedly reduced AHR to inhaled methacholine of 50 mg/ml in OVA-challenged mice as indicated by Penh index ([175.92 ± 21.38]% *vs.* [583.74 ± 59.26]%, $P < 0.001$). Meanwhile, administration of MSCs starkly decreased the numbers of macrophages ([33.86 ± 5.71] × 10^4/ml *vs.* (52.19 ± 7.12) × 10^4/ml, $P < 0.001$), eosinophils ([21.46 ± 3.51] × 10^4/ml *vs.* [73.59 ± 9.62] × 10^4/ml, $P < 0.001$), lymphocytes ([19.82 ± 2.94] × 10^4/ml *vs.* [33.59 ± 4.27] × 10^4/ml, $P < 0.001$), and neutrophils ([12.61 ± 0.92] × 10^4/ml *vs.* [17.52 ± 2.76] × 10^4/ml, $P < 0.001$) in BALF [Figure 1A]. H&E and PAS staining demonstrated that MSCs transplantation remarkably suppressed inflammatory cell infiltration into peribronchial and perivascular connective tissues (1.97 ± 0.31

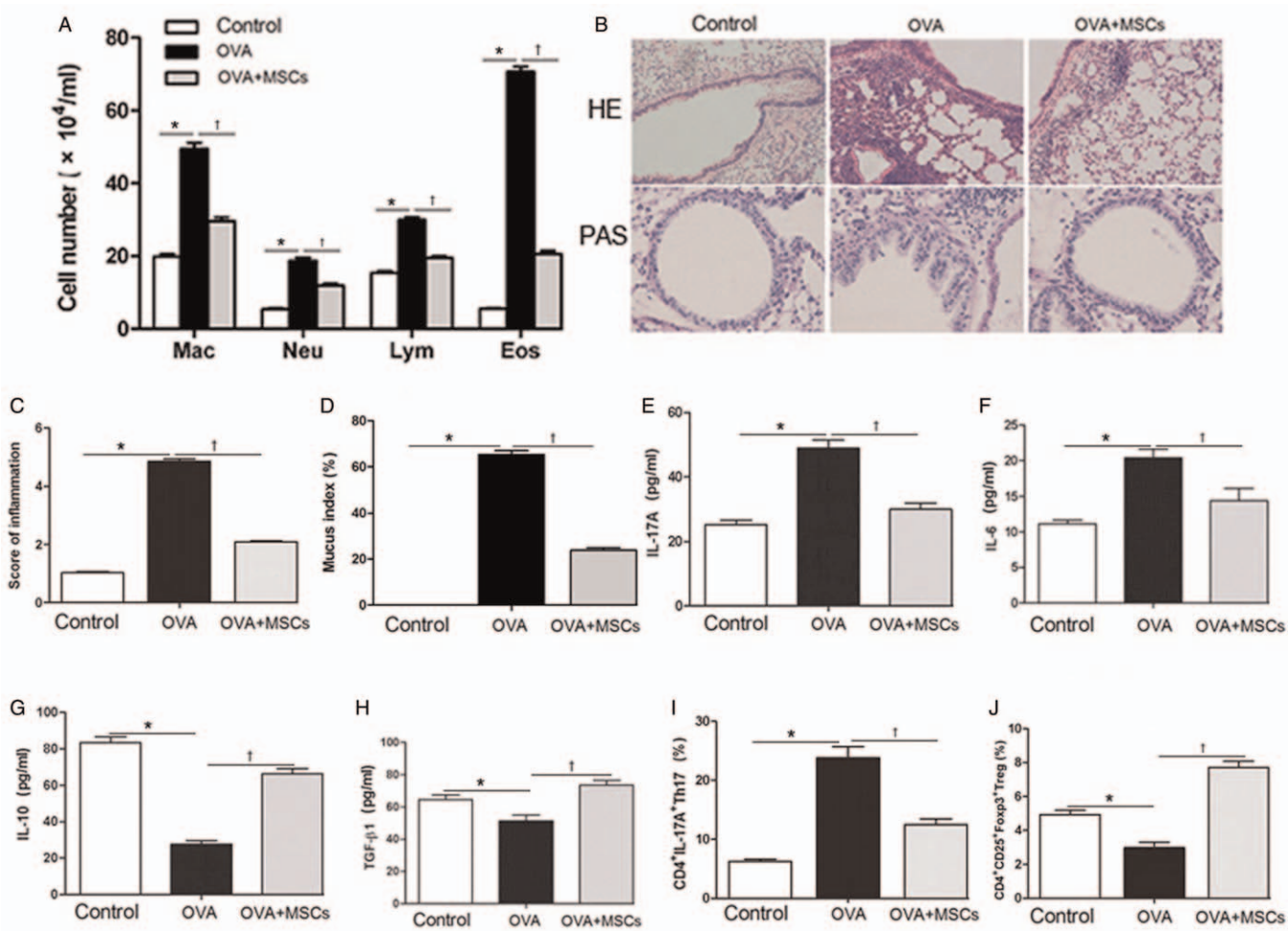

Figure 1: (A) MSCs administration reduced inflammatory cell infiltration of OVA-challenged mice in BALF. (B–D) Effects of MSCs administration on histopathological changes of the lung. Representative photographs of lung sections that were stained with HE (B, original magnification, × 200) or PAS (B, original magnification, ×400). The inflammation score for the peribronchi was determined (C). Mucus production was evaluated with an image analyzer. The mucus index was calculated as the percentage of mucus-positive area in the whole bronchial epithelium (D). MSCs administration regulated the secretion of cytokines IL-17A (E), IL-6 (F), IL-10 (G), and TGF-β1 (H) in BALF of OVA-induced asthma. MSCs administration balanced Th17 (I) and Treg (J) percentages in splenocytes of OVA-induced asthma. $^*P < 0.001$, OVA group compared with control group. $^\dagger P < 0.001$, OVA + MSCs group compared with OVA group. BALF: Bronchoalveolar lavage fluid; Eos: Eosinophils; Foxp3: Forkhead box P3; HE: Hematoxylin and eosin; IL: Interleukin; Lym: Lymphocytes; Mac: Macrophages; MSCs: Mesenchymal stem cells; Neu: Neutrophils; OVA: Ovalbumin; PAS: Periodic acid-Schiff; PBS: Phosphate buffer saline; TGF-β1: Transforming growth factor β1; Th17: T-helper 17; Treg: Regulatory T cell.

vs. 5.13 ± 0.98, $P < 0.001$; Figure 1B and 1C) and dramatically attenuated mucus production (mucus index: [22.54 ± 3.26]% *vs.* [67.16 ± 6.24]%, $P < 0.001$; Figure 1B and 1D). Furthermore, treatment with MSCs obviously decreased BALF IL-17A ([32.74 ± 4.97] pg/ml *vs.* [48.62 ± 7.21] pg/ml, $P < 0.001$; Figure 1E) and IL-6 ([14.71 ± 2.18] pg/ml *vs.* [22.69 ± 3.28] pg/ml, $P < 0.001$; Figure 1F) levels, while IL-10 ([68.41 ± 8.22] pg/ml *vs.* [26.37 ± 3.13] pg/ml, $P < 0.001$, Figure 1G), and TGF-β1 ([76.19 ± 7.81] pg/ml *vs.* [51.36 ± 6.62] pg/ml, $P < 0.001$; Figure 1H) concentrations were increased in comparison with the asthma group. Flow cytometric analysis of splenocytes revealed markedly elevated population of $CD4^+IL\text{-}17A^+Th17$ cells in OVA group compared with the normal control group ([26.14 ± 4.21]% *vs.* [7.22 ± 0.74]%, $P < 0.001$), but this Th17 induction was dramatically decreased in the MSCs treatment group ([15.97 ± 2.58]% *vs.* [26.14 ± 4.21]%, $P < 0.001$; Figure 1I). Decreased percentage of $CD4^+CD25^+Foxp3^{+-}$ Treg cell was found in asthmatic mice compared with the control group ([3.22 ± 0.39]% *vs.* [4.86 ± 0.49]%, $P < 0.001$; Figure 1J), while significant increase of these cells was observed in the MSCs treatment group compared with the asthmatic OVA group ([7.81 ± 1.13]% *vs.* [3.22 ± 0.39]%, $P < 0.001$; Figure 1J).

Asthma is characterized by intermittent airway obstruction, bronchial hyperreactivity and chronic inflammation with airway structural changes. Varieties of medical remedies for asthma relief have diverse side effects and limited efficacies after long-time treatment. Thus, new effective therapeutic approach is desirable. In recent decades, MSCs have been shown to interact with immune cells and regulate inflammatory responses, constituting potential candidates for asthma therapy. MSCs migrate preferentially to inflammatory sites after tissue damage. Studies have demonstrated that MSCs migrate to lung tissues and suppress histopathological alterations in asthmatic mice.[4] In our present study, treatment with MSCs in chronic asthma models reduced airway inflammation and remodeling, as well as bronchial hyperresponsiveness.

Migration of inflammatory cells, especially eosinophils and lymphocytes, into the lung is critical to allergic airway inflammation. Our present results showed that MSCs remarkably decreased eosinophil numbers in BALF and lung tissue. Although the Th2 pathway likely plays an important role in allergic asthma, there are shortcomings to the notion that Th2 inflammation is the only mechanism involved in asthma. Recently, Treg cells and Th17 cells have been described as two distinct subsets from Th1 and Th2 cells, which have obvious change in bronchial asthma patients. Higher levels of Th17 cells and lower level of Treg cells can promote the occurrence of asthma. It is reported that MSC-mediated protective effects are related to activated Treg and high IL-10 production in allergic airway inflammation. In the present study, administration of MSCs markedly promoted Treg differentiation and IL-10 production in BALF, but decreased Th17 cell differentiation, with concomitantly reduced infiltration of inflammatory cells, airway remodeling, and bronchial hyperresponsiveness.

IL-17A, a Th17 cell marker, is increased in BALF from asthmatic mice. It plays critical roles in eosinophil, neutrophil and macrophage infiltration, promoting Th2 type inflammatory reactions and inducing severe asthma. In our present study, asthmatic mice treated with MSCs showed markedly decreased IL-17A levels but increased IL-10 levels, and inflammatory cell infiltration, airway remodeling, and bronchial hyperresponsiveness were attenuated.

Th17 and Treg differentiation were interacted with TGF-β, and Th17/Treg are regulated in a reciprocal way. IL-6 induces Th17 cell generation but suppresses Treg differentiation. Tregs inhibit AHR and Th2 mediated inflammation via induction of TGF-β in the airway. In the present study, administration of MSCs suppressed Th17 differentiation but enhanced Treg differentiation. This may result from decreased IL-6 but increased induction of TGF-β.

It has been reported that IL-10 induces Treg differentiation and activation in the lung; meanwhile, IL-10 decreases Th17 cell function.[5] Bone marrow-derived MSCs could inhibit the production of Th17 cells via IL-10. As shown above, MSCs enhanced $CD4^+CD25^+Foxp3^+$Tregs, and increased IL-10 level in BALF, with concomitant suppression of Th17 cell functions, and attenuated airway inflammation and remodeling.

In summary, this work revealed that administration of MSCs attenuates OVA-induced murine asthma exacerbation, reducing airway inflammation and remodeling likely by regulating imbalanced Th17/Treg responses.

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Conflicts of interest

None.

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