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# The effects of bone marrow-derived mesenchymal stem cells on ovalbumin-induced allergic asthma and cytokine responses in mice

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**ABSTRACT**

***Objective(s)***: Allergic Asthma is an inflammatory disease of the lungs that is characterized by increased infiltration of leukocytes into the airways, limiting the respiratory function. Studies suggest that a defective general regulatory system against inflammation could be a significant factor in allergic asthma. It has been shown that Mesenchymal stem cells (MSCs) have a cellular immunosuppressive therapeutic potential for inflammatory disorders. We investigated whether administration of MSCs during allergen challenge would affect the underlying mechanisms in allergic airways inflammation.

***Materials and Methods:*** Fifty mice were used in five control and experimental groups; the experimental mice sensitized by intraperitoneal injection of OVA and aluminum hydroxide emulsion on days 0, 7, and 14, were then challenged intranasally with OVA or sterile PBS on days 14, 25, 26, and 27. Before allergen challenge on day 14, experimental mice received tail vein injection of MSCs in PBS, whereas control mice received PBS alone. Cytokine and IgE analyses were carried out using lung washes as well as serum samples.

***Results:*** Our, results showed that MSCs significantly reduced total cells and eosinophilia and serum OVA-specific IgE concentration in OVA-sensitized and challenged mice. Also, results showed that MSCs markedly inhibited expressions of Th2 and Th17 cytokines and elevated levels of Treg cytokines.

***Conclusion:*** we found that administration of MSCs could be used as a potential therapeutic approach for allergic asthma.

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## Introduction

Allergic Asthma is an inflammatory disease of the lungs that is characterized by increased infiltration of leukocytes, especially eosinophils, into the airways, limiting respiratory function. This inflammation leads to bronchial constriction, airway hyperresponsiveness (AHR) increment, and massive mucus production (1). The prevalence of allergic asthma is increasing worldwide, and it has become a significant cause of health challenge especially in developed countries (2). Inhaled β2-agonists and inhaled or oral glucocorticosteroids are common medications for treating the disease, but they cannot be used for long periods of time because of frequently occurring side effects and they can't change the main pathogenesis of the problem (3). Therefore, there is a necessity for improved treatment strategies for providing higher life quality for asthmatic patients. The underlying process that causes and maintains the asthmatic inflammatory response is an imbalance between T helper 1 (Th1) and T helper 2 (Th2) immune responses. This imbalance is biased towards an increased Th2 response. The Th2 mediated cytokines are correlated with allergic reactions and eosinophilia, which are directly linked to the inflammatory process of allergic asthma (4). Although this hypothesis still could be valid, therapies focused on inhibiting Th2 mediated immune response have generally resulted in impartial success. Studies suggest that a defective general regulatory system against inflammation could be a significant factor in allergic asthma and perhaps other allergic disorders. The studies on immunoregulatory mechanisms have resulted in a specific subset of regulatory T cells (CD4+CD25+Foxp3+cells) that suppress inflammatory responses, promoting immune tolerance (5, 6). Provided evidence shows that the diminished levels of Tregs and their activities, resulted in increased airway hyperreactivity, which links Tregs to airway physiology (7). With regards to the capacity of Tregs to suppress immune-mediated disease, enhancing Treg function in patients represents a promising therapeutic strategy for treating allergic disorders, including allergic asthma. Mesenchymal stem cells (MSCs) are ubiquitous multipotent cells that are abundant in adult bone marrow and adipose tissue (6). MSCs have gained significant interest due to their pliability to develop into almost any cell type (8), making them attractive for regenerative medicine. MSCs also have potential as a cellular immunosuppressive therapy for inflammatory disorders, such as systemic lupus erythematosus (9, 10), rheumatoid arthritis(11, 12), inflammatory bowel disease (13, 14), and graft-versus-host disease (15, 16). Protective roles for MSCs

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have also been described in models of acute lung injury, including pulmonary fibrosis (17) and more recently allergic rhinitis (18). Despite extensive information on the immune functions of MSCs and their potential to treat inflammatory diseases, the precise mechanism by which MSCs mediate protection is less clear, so, MSC therapy has not reached the clinic for the treatment of allergic inflammation (19). In the present study, we investigated whether administration of MSCs during allergen challenge would affect the underlying mechanisms in allergic airways inflammation, including Th2, Treg, Th17, and proliferation of allergen-specific cells in a mouse model.

## Materials and Methods

### *Animals*

The Female inbred BALB/c mice were housed and maintained in the animal facilities of Biotechnology Institute of Urmia University. The animals were allowed to rest for at least one week before entering the experiments at 8–12 weeks of age. The mice were exposed to a 12-hr/12-hr light/dark cycle (30–60 lux in cages), 20±1 ˚C room temperature, and 40–60% relative humidity. The animals were given pelleted food and tap water. All studies conformed to the principles for laboratory animal research outlined by the Animal Welfare Act and were approved by the Urmia University Animal Care and Use Committee (ethical ref. no.: 94-115a). In this study, the 50 female BALB/c mice were divided into 5 groups of ten mice each, and each experiment was repeated three times.

### *Antigen challenge*

To elicit a bronchial allergic response in female mice (8- to 12-weeks old), OVA sensitization and challenge were used, whereas male BALB/c mice were the source of bone marrow-derived MSCs (20, 21). Briefly, mice were sensitized by intraperitoneal injection of 300 µl of OVA (100 µg/ml) (grade V; Sigma Aldrich, St. Louis, MO, USA) and aluminum hydroxide (1 mg/ml) (Sigma Aldrich, St. Louis, MO, USA) emulsion on days 0, 7, and 14. Mice were challenged intranasally with 30 µl OVA (50 µg/ml) or sterile PBS (sham) on days 14, 25, 26, and 27. Control animals were intraperitoneally injected with the same amount of aluminum hydroxide mixed with a PBS solution (Figure 1). In this study dexamethasone (2 mg/kg) was used as positive control and administered 1 hr prior to OVA challenge intraperitonally. In all cases, mice were killed by anesthetizing with ketamine (Alfasan, Netherlands) and xylazine (Alfasan, Netherlands) 100 mg/kg and 10 mg/kg, respectively, followed by exsanguination.

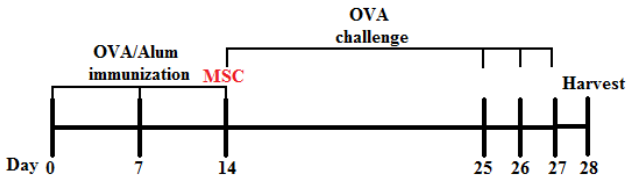


**Figure 1.** Timeline of the experimental protocol

### *Isolation and culture of bone marrow-derived mesenchymal stem cells*

MSCs were isolated from the bone marrow of male BALB/c mice as described previously (22) with minor modifications. Briefly, bone marrow cells were collected by the flushing method with phosphate-buffered saline (PBS) supplemented with 2% fetal bovine serum (FBS) (GIBCO, USA). The collected cells were cultured and maintained in T25 flasks containing Dulbecco's modified Eagle's medium (DMEM) (GIBCO, USA) supplemented with 15% FBS, 100 IU/ml penicillin, and 100 µg/mL streptomycin (GIBCO, USA). The culture medium was changed at day 3 to remove non-adherent cells. The medium was then replaced two times each week. The adherent cells were grown, detached with 0.25% trypsin-EDTA, and passaged for 2–3 weeks. Subsequent passage and cell seeding were performed at a density of $1\times10^6$ cells/flask. Passage 4 or 5 of MSCs was used for characterization analysis. MSCs phenotype was verified by surface marker expression of CD29, CD44, CD90, Sca-1, CD34, CD45, CD14, and CD11b using PE- or FITC-conjugated antibodies (BD Biosciences, San Jose, NJ, USA). Flowcytometric analyses were performed using a Partec PAS flowcytometer (Partec GmbH, Münster, Germany). FloMax software (version 2.5.1) was used for data analysis.

### *MSC treatment*

Immediately, before allergen challenge on day 14, experimental mice received tail vein injection of $2 \times 10^6$ MSCs in 100 µl PBS, whereas the control group received PBS alone.

### *Bronchoalveolar lavage fluid (BALF) cell and cytokine analysis*

Just after the mice were exsanguinated, the lungs were cannulated with a 20-gauge IV catheter and BALF was collected from the left middle or diaphragmatic lung lobe (23). For cytokine analysis, lungs were gently washed once with 500 µl of PBS containing 1% FBS (GIBCO, USA). For analysis of cellular infiltration, lungs were washed twice with 1 ml of PBS containing 1% FBS, the samples were stored at -80 °C for cytokine analysis and for microscopic review, the samples were centrifuged, resuspended, and applied Giemsa-Wright (Sigma, USA) differential staining.

### *Serum OVA-specific IgE assay*

Blood was collected via cardiopuncture of mice under ketamine/xylazine anesthesia. Serum samples were obtained by centrifugation and stored at -80 °C. The serum was diluted 1:25 in assay diluent buffer and OVA-specific serum IgE was measured using an EIA kit (Cayman, Ann Arbor, MI, USA), according to the manufacturer's protocol. Optical density was measured at 450 nm by a microplate reader (Becton-Dickinson, New Jersey, USA).

*Cytokine assay*

IL-4, IL-5, IL13, IL-17, IL-10, and TGF-β from BALF and serum samples were analyzed using LEGEND MAX™ ELISA kits (BioLegend, London, UK) according to manufacturer's instructions (24).

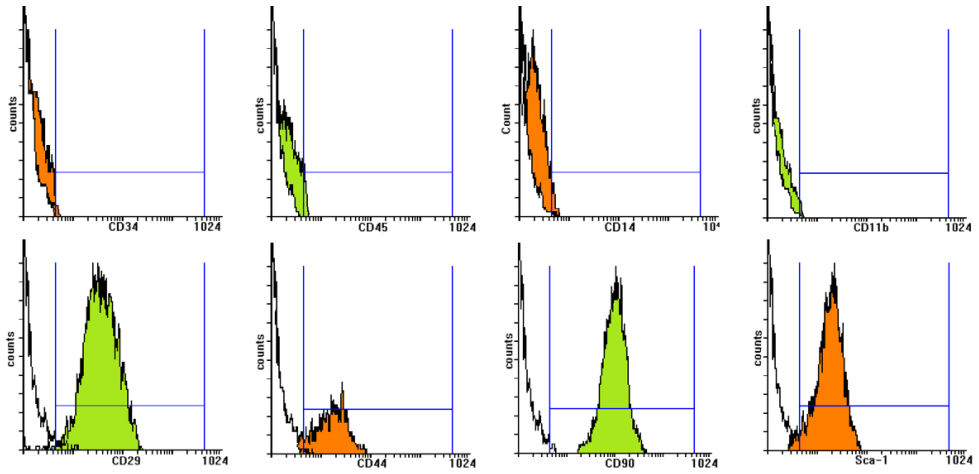


**Figure 2.** Flow cytometric analysis of cell surface markers of mice bone marrow derived MSCs. The respective isotype control is shown as white. CD29 (92.38%), CD44 (79.66), CD90 (96.27%), Stem cell antigen (Sca)-1 (82.12%), CD34 (0.8%), CD45 (0.5%), CD14 (0.6%), and CD11b (0.5%)

### *Proliferation assay*

Proliferation assay was carried out as described previously (25) with minor modifications. Briefly, MSCs ($1\times10^3$ cells) were plated in round-bottom 96-well plates (Thermo, USA) in a total volume of 0.2 ml of DMEM and one day before splenocytes proliferation assays. Splenocytes were isolated from OVA-sensitized and control BALB/c mice spleens. Erythrocytes were removed using a lysing buffer ($NH_4Cl$, 0.84%) with incubation for 1 min, followed by two washes in RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine. Splenocytes ($1\times10^5$ cells) were added to the MSC culture after removing the DMEM. T cell proliferation assays were performed in a total volume of 0.2 ml of complete RPMI 1640. Triplicate cultures were incubated at 37 °C for 4 days in the presence of 200 µg/ml OVA. The medium was removed and MTT reagent (Sigma-Aldrich, St. Louis, MO, USA) (0.5 mg/ml) was added to the wells and incubated for 2 hr at 37 ˚C in 5% $CO_2$. After incubation, the MTT solution was removed and 100 µl/well of dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA) was added. The absorbance of the reduced form of MTT was measured at 550 nm and 650 nm (background) by a plate reader (Becton-Dickinson, New Jersey, USA).

### *Data analysis*

Data were summarized as mean±SD. One-way ANOVA and Tukey's *post hoc* were performed using GraphPad Prizm TM version 5.00 (GraphPad, San Diego, CA, USA). The statistical significance value was set at $P \leq 0.05$.

## Results

### *Bone marrow derived MSCs characterization*

Homogenous bone marrow derived MSCs were obtained from BALB/c mice, after 5 passages *in vitro*. Flowcytometric analysis, confirmed these cells are CD29, CD44, CD90, and Sca-1 positive and CD34, CD45, CD14, and CD11b negative (Figure 2).

### *BALF total cell and eosinophilia*

OVA-induced a significant elevation of total cell and eosinophils counts in BALF in comparison to the untreated group ($P\leq0.01$). OVA-sensitized and challenged mice treated with MSCs during sensitization displayed significantly reduced total cell and eosinophil counts ($P\leq0.05$). These reductions by MSCs were comparable to the dexamethasone treatment, as a positive control for anti-inflammatory effects (Figure 3).

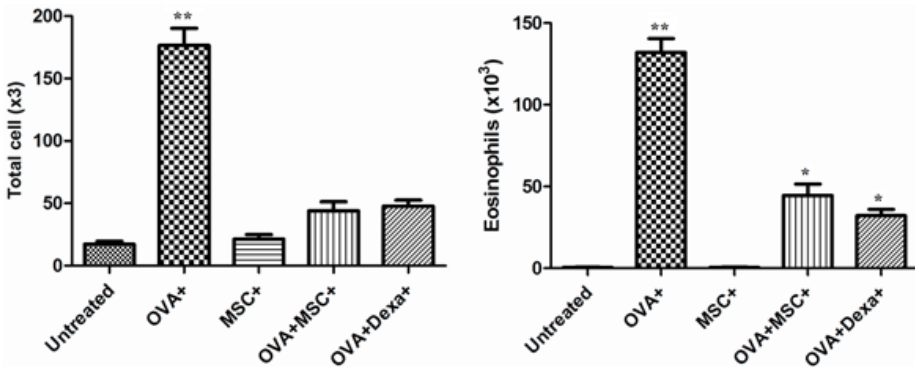


**Figure 3.** Evaluation of the effect of MSCs treatment on total numbers of BAL cells and BAL eosinophils, untreated: negative control; OVA+: OVA-sensitized and OVA-challenged; MSC+: not sensitized, not challenged and MSC administered; OVA+MSC+: OVA-sensitized, OVA-challenged, and MSC administered; OVA+Dexa+: OVA-sensitized, OVA-challenged and dexamethasone administered, the results are presented as mean±SD. *: $P\leq0.05$, **: $P\leq0.01$

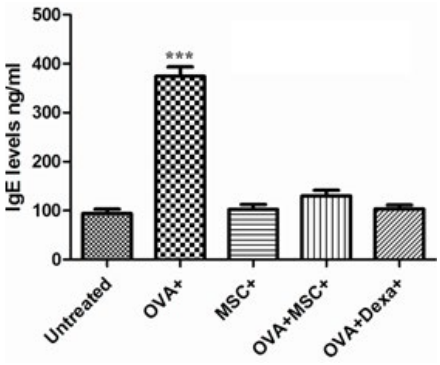


Figure 4. Evaluation of the effect of MSCs treatment on serum IgE levels, levels of OVA-specific IgE were measured by ELISA in serum 24 hr after the last OVA challenge, untreated: negative control; OVA+: OVA-sensitized and OVA-challenged; MSC+: not sensitized, not challenged and MSC administered; OVA+MSC+: OVA-sensitized, OVA-challenged and MSC administered; OVA+Dexa+: OVA-sensitized, OVA-challenged and dexamethasone administered, the results are presented as mean±SD. ***: $P \leq 0.0001$

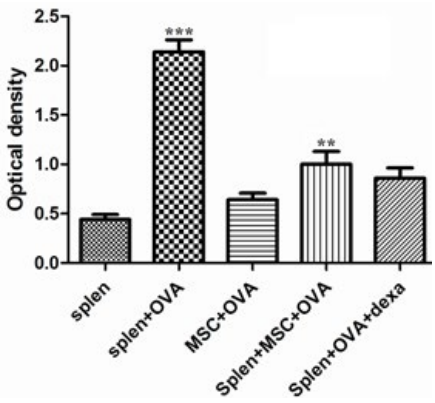


**Figure 6.** The effect of MSCs on OVA-induced proliferation of splenocytes, untreated: negative control; OVA+: OVA-sensitized and OVA-challenged; MSC+: not sensitized, not challenged and MSC administered; OVA+MSC+: OVA-sensitized, OVA-challenged, and MSC administered; OVA+Dexa+: OVA-sensitized, OVA-challenged and dexamethasone administered, the results are presented as mean±SD. **: $P \leq 0.01$, ***: $P \leq 0.0001$

### *Serum IgE levels*

Level of OVA-specific IgE in serum was measured by ELISA, 24 hr after the last OVA challenge. Indeed, our results showed that serum-derived OVA-specific IgE levels were significantly increased following OVA application ($P \leq 0.0001$). Bone marrow MSCs treatment resulted in a significant decrease of OVA-induced elevation of OVA-specific IgE concentration in both serum and BALF ($P \leq 0.0001$) (Figure 4).

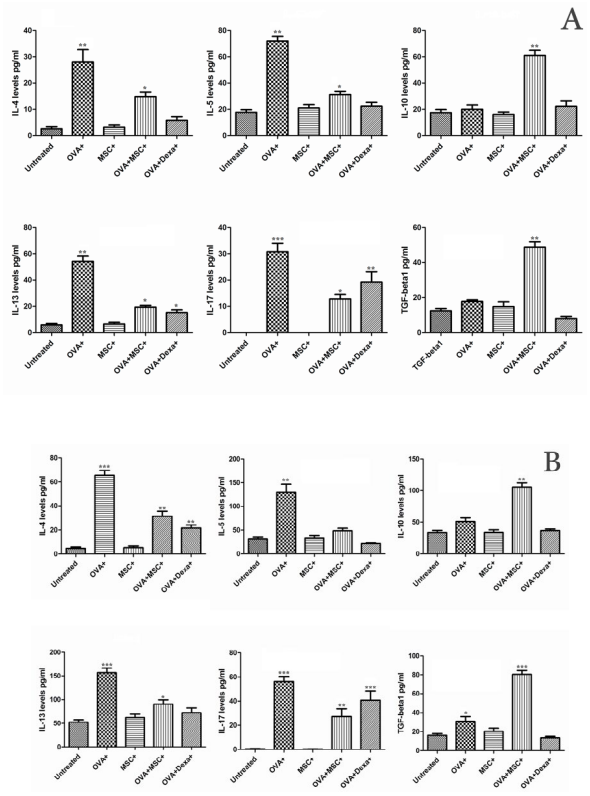


**Figure 5.** Evaluation of the effect of MSCs treatment on BALF (A) and serum (B) cytokines levels, untreated: negative control; OVA+: OVA-sensitized and OVA-challenged; MSC+: not sensitized, not challenged and MSC administered; OVA+MSC+: OVA-sensitized, OVA-challenged and MSC administered; OVA+Dexa+: OVA-sensitized, OVA-challenged, and dexamethasone administered the results are presented as mean±SD.*: $P \leq 0.05$, **: $P \leq 0.01$

### *Cytokine response*

OVA-sensitised and challenged mice showed increased levels of IL-4, IL-5, IL-13, and IL-17 in both serum and BALF ($P \leq 0.01$), but IL-10 and TGF-β did not show any significant changes ($P \leq 0.05$). Elevations in Th2 cytokines highlight allergic reaction, and increased IL-17 levels were the result of inflammation. When OVA-sensitized and challenged animals were treated with MSCs, IL-4, IL-5, IL-13, and IL-17 levels were decreased and IL-10 and TGF-β levels in serum and BALF were increased significantly ($P \leq 0.01$), (Figure 5).

### *Splenocyte proliferation*

Bone marrow-derived MSCs were co-cultured with OVA-sensitized mice splenocytes to determine whether MSCs could affect the proliferation of splenocytes in response to the OVA-antigen. Splenocyte proliferation levels were significantly increased following OVA application. On the other hand, results showed that MSCs induced an inhibitory effect on OVA-induced proliferation of splenocytes ($P \leq 0.001$). MSCs induced inhibition was comparable to dexamethasone inhibitory effect (Figure 6).

## Discusion

Allergic asthma is a chronic inflammatory disorder distinguished by airway inflammation, reversible airway obstruction and bronchial hyperresponsiveness (1). An imbalance of the Th1/Th2 immune responses towards Th2 is thought to play an important role in the development of allergic asthma. This disorder may result from deficiencies in the regulatory T cells that control the lungs immune responses (5). Larche reported that Treg cells were decreased in the bronchial fluid of asthmatic children compared with control subjects (7). Many studies have demonstrated that Treg cells play a pivotal role in the maintenance of tolerance in the immune system (26), for example by helping to prevent autoimmune diseases and allergy

(8). Considering the ability of regulatory T cells to suppress immune responses, enhancing Treg function in patients with immune-mediated diseases is an attractive potential therapy for treating allergic asthma (27, 28). Nowadays corticosteroids are the commonly administered therapeutic in allergic cases (1), however, these drugs did not change the main pathogenesis of allergic disorder and prolonged use of corticosteroids, especially at higher doses, has been accompanied by concerns about both systemic and local side effects (29).

In the present study, we demonstrated the inhibitory effect of MSCs on airway inflammation using a mice model of allergic asthma. The mice were sensitized with OVA and the results were compared of dexamethasone administration. Our results showed that MSCs significantly reduced total cells and eosinophilia as well as serum OVA-specific IgE concentration in OVA-sensitized and challenged mice. To investigate the underlying immunoregulatory mechanism of MSCs on allergic asthma, we determined serum levels of cytokines associated with airway inflammation. Our findings showed that MSCs markedly inhibited induction of Th2 and Th17 cytokines (including IL-4, IL-5, IL-13, and IL-17A) and elevated levels of Treg cytokines (including TGF-β and IL-10) and that proliferation of OVA-sensitized and challenged splenocytes was decreased in presence of MSCs *in vitro*. However, unlike MSCs, dexamethasone suppressed Th2 (IL-4, IL-5, and IL-13) responses as well as Treg (TGF-β and IL-10) responses (Figures 4, 5), which resulted in the suppression of allergic reaction, temporally. However, dexamethasone did not affect the Th17 cytokine response, Th2, or Treg cells. These findings suggest that MSCs may offer clinical advantages over corticosteroids.

MSCs are increasingly being found to have potent anti-inflammatory effects in a wide range of inflammatory and immune-mediated disease models (11, 30). It has recently been demonstrated that systemic administration of either syngeneic or allogeneic BMSCs in a ragweed-induced model of allergic airways disease also led to a decrease in airways inflammation as assessed by BAL circulating antibodies, cytokine levels, and histology. The release of TGF-β from the MSCs stimulated an increase in T regulatory cells in the lungs with subsequent suppression of allergic airways inflammation (20). Thus, MSCs administration can decrease airways inflammation, when given during either immunization or challenge (20). Interestingly, because of the presence of several cytokines and chemokine receptors on MSCs, the functions of these cells can be influenced by the tissue microenvironment through cross-communication with specific ligands (19). This highlights an appreciation of MSCs to feel or sense their microenvironment and respond in different ways to suppress inflammatory and immune responses.

Allergic asthma is primarily mediated by Th2 cells, which secrete the typical cytokines IL-4, IL-5, and IL-13. IL-4 has a key role in the differentiation of Th2 from Th0 cells and may be important in initial sensitization to allergens. It is also important for class switching of B cells from IgG to IgE secretion (1). Similar to IL-4, IL-13 mainly induces IgE secretion but does not promote Th2 cell differentiation. IL-5 has a critical role in airway eosinophilia; it regulates different aspects of eosinophil behavior, including growth, maturation, adhesion, secretion, and apoptosis (31). In contrast to Th2 cells, Treg cells have an opposite effect. TGF-β and IL-10 are predominant cytokines produced by Treg cells. Treg is involved in regulation of Th2 and Th17 cell responses and IgE synthesis to restrain the progress of asthma. Accordingly, examination of Th2 cytokine levels is an important index in the evaluation of asthma (32).

It is well documented that IL-17 could recruit neutrophils to the respiratory system (33). Studies showed that Th17 cells together with Th2 cells promoted the infiltration of eosinophils and neutrophils to the airways and airway hyper-reactivity (AHR) (34). Previous studies found that in the peripheral blood of moderate to severe asthma patients, Th17 cells, IL-17, and transcriptional factor RORγt mRNA were elevated (20). Other studies show that IL-17 concentration in sputum positively associated with the AHR of the patient's airway (35).

As Treg cells have the ability to suppress the proliferation and function of other T cells (including Th1, Th2, and Th17), researchers paid attention to the role of Treg cells played in allergic asthma (35). They demonstrated that although Treg cells could be recruited to the lungs when allergens triggered the allergic reaction, the population of Treg cells lost their suppressive function. It means that Treg cells in the lungs of patients with asthma had a deficient function, and more research confirmed that the deficiency of Treg cells in asthma led to imbalanced immunological regulation (36). So, either hyperactivity of Th17 cells or Treg cells deficiency were altogether involved in the immunological pathogenesis of asthma (37). Hou *et. al.* reported that this imbalance existed in the periphery blood and draining lymph nodes in asthma and that the imbalance also occurred in the lung tissue (38). Therefore, it could be a potential beneficial remedy for asthma treatment if the imbalanced status of Th1/Th2 and Treg/Th17 cells can be reversed.

Altogether, under physiological conditions, Th0 cells differentiate to Th1, Th2, Th17 and Treg cells proportionally and keep their numbers in a relative balance. When this balance is disturbed, diseases will occur. It has been reported that the imbalanced Th1/Th2 cells and Treg/Th17 exist in patients with allergic asthma (33). Imbalances in Th17/Treg cells and Th1/Th2 cells play an important role in asthma. An imbalance in Th1/Th2 cells may trigger allergic asthma, but an imbalance in Th17/Treg cells results in exacerbated allergic asthma.

## Conclusion

We found that transplantation of bone marrow-derived MSCs could prevent allergic airway inflammation via correction of Th1/Th2 and Treg/Th17 cell imbalance in a mice model. MSCs could be utilized as a potential therapeutic approach for allergic asthma; however, more studies are necessary to elucidate other mass events of MSCs transplantation in allergic asthma disorder.

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## Conflicts of Interest

The authors declare that they have no conflicts of interest.

## Results

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