

RESEARCH ARTICLE

Intratracheal transplantation of bone marrow-derived mesenchymal stem cells reduced airway inflammation and up-regulated CD4⁺CD25⁺ regulatory T cells in asthmatic mouse

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Abstract

Mesenchymal stem cells attenuate the severity of lung injury due to their immunomodulatory properties. The effect of bone marrow-derived mesenchymal stem cells on asthma is seldom reported. We have examined the effect of BMSCs on airway inflammation in asthma. Forty female BALB/c mice were equally randomised into PBS group, BMSCs treatment group, BMSCs control group and asthmatic group. Reactivity of the airway to acetylcholine was measured by barometric plethysmography. Cytokine profiles of bronchoalveolar lavage fluid and serum were determined by enzyme-linked immunosorbent assay. Morphometric analysis was done with haematoxylin and periodic-acid Schiff staining. Engraftment of BMSCs in asthmatic mice significantly decreased the number of eosinophils and mononuclear cells in bronchoalveolar lavage fluid and the airway ($P < 0.05$). Both goblet cell hyperplasia and responsiveness to acetylcholine were significantly reduced in BMSCs treatment groups. Moreover, BMSCs engraftment caused significant increases the ratio of Treg in pulmonary lymph node and interleukin-10 (IL-10) and interleukin-12 levels in BALF and serum. We conclude that BMSCs engraftment ameliorated airway inflammation and improved lung function in asthmatic mouse and the protective effect might be mediated by upregulating Treg and partly involved with increasing IL-10.

Keywords: asthma; bone marrow mesenchymal stem cells; IL-10; regulatory T cells; lung inflammation

Introduction

Asthma is a common disorder affecting ~300 million people worldwide, with a prevalence of 1–18% varying among countries and age groups (Beasley, 2004; Masoli et al., 2004). Although most patients can control the disease, 20% of patients remain uncontrolled (Rabe et al., 2004). In these cases, airway remodelling may not be prevented by or fully responsive to currently available treatments (Holgate and Polosa, 2006). Inflammatory and immune responses are actively involved in resistance during the treatment of asthmatic attacks; however, the mechanism is unclear. Bone marrow-derived mesenchymal stem cells (BMSCs), non-haematopoietic adult stem cells residing in the bone marrow,

have the ability to modulate immune responses and to promote tissue repair (Anjos-Afonso et al., 2004; Yamada et al., 2004; Wang et al., 2005).

Mesenchymal stem cells (MSCs) can differentiate into multiple cell types both in vitro and in vivo (Anjos-Afonso et al., 2004; Yamada et al., 2004; Wang et al., 2005). In lung disease models, MSCs differentiate into type I and II epithelial cells, and lung fibroblasts (Kotton et al., 2001; Ortiz et al., 2003; Rojas et al., 2005). The rate of differentiation of MSCs in airway epithelium and alveolar after engraftment of MSCs is much higher in myelosuppression mice than that in non-bone marrow depression mice. However, engraftment of MSCs can attenuate the severity of lung injury and myocardial infarction in low

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Abbreviations: AHR, airway hyperresponsiveness; ALI, acute lung injury; BALF, bronchoalveolar lavage fluid; BMSCs, bone marrow-derived mesenchymal stem cells; IFN- γ , interferon-gamma; IL, interleukin; MCh, methacholine; MSCs, mesenchymal stem cells; OVA, ovalbumin; PAS, periodic-acid Schiff; TGF- β , transforming growth factor- β ; TNF- α , tumour necrosis factor-alpha; Treg, regulatory T cell; WBP, whole body plethysmography

efficiency of MSCs differentiation (Gupta *et al.*, 2007; Iso *et al.*, 2007; Ortiz *et al.*, 2007; Xu *et al.*, 2007), indicating that protective effects of MSCs are not attributed to engraftment itself. Recent studies have shown that MSCs can modulate the activity of innate and adaptive immune cells, such as dendritic cells, T cells and B cells (Di Nicola *et al.*, 2002; Beyth *et al.*, 2005; Jiang *et al.*, 2005), and these activities are mediated by direct cell-to-cell interactions and soluble factors secreted from MSCs.

Asthma is an immune-mediated disease characterised by a shift toward the TH2-cytokine profile and inflammation with eosinophilic infiltration. However, only a few studies indicate that transplantation of BMSCs result in the substantial reduction in lung inflammation in asthmatic mice. Moreover, the beneficial mechanism of BMSCs in asthma mouse models is not completely understood (Kong *et al.*, 2008). We hypothesised that BMSCs played an important role in modulating inflammation and local immunity of the lung in asthma. Thus, we aimed to test the effects of BMSCs on the inflammation and changes of Treg and cytokines in an ovalbumin (OVA)-induced asthmatic mouse model. Intrapulmonary route of administration was used to maximise the efficiency of delivery of BMSCs to the lung (Furlani *et al.*, 2009). The results showed that intrapulmonary treatment with BMSCs had both histological and functional advantages in asthmatic mice. Furthermore, this benefit might be mediated by the upregulation of Treg and protective cytokines in asthmatic mice.

Materials and methods

Animals

BALB/c mice were purchased from the Shanghai Laboratory Animal Center. Mice were bred and maintained in the animal facility at the Second Military Medical University, Shanghai, P.R. China. The care and use of the animals were in compliance with National Institute of Health Guide, with the approval of the Scientific Investigation Board of Second Military Medical University, Shanghai.

Isolation and expansion of BMSCs

BMSCs were obtained from male BALB/c mice at 5–6 weeks of age. Murine bone marrow cells were collected and cultured as described previously (Phinney, 2008). Briefly, mice were sacrificed by decapitation, BMSCs were isolated through the medullary cavity of mouse femurs by flushing with Dulbecco's modified Eagle's medium (DMEM; Gibco, Manassas, MD) containing 1% penicillin–streptomycin (Gibco, BRL, Grand Island, NY). The cells were washed once with DMEM and plated at 2×10^6 /mL in a 100-mm cell culture dish in DMEM media containing 10% foetal calf

serum (FBS; Gibco) supplemented with 2 mM L-glutamine (Gibco), non-essential amino acids (Gibco) and pyruvate (Gibco). Cells were cultured at 37°C in air plus 5% CO₂. After 72 h of culture, the non-adherent cells were removed by a medium change. Cells were passaged every 3 or 4 days by trypsinisation, and three to five passages were used for *in vitro* experiments. Cells were checked daily using an inverted phase contrast microscope.

Adipogenic differentiation assay of BMSCs

BMSCs from passage 5 were cultured in 6-well plates in a complete culture medium. On the following day, at 100% confluence, the medium was replaced with adipogenic induction medium [DMEM supplemented with 10% FBS, 1% penicillin–streptomycin, 2 mM glutamine, 10 µg/mL insulin (Sigma), 0.5 mM/L 3-isobutyl-1-methylxanthine, 0.1 mM/L indomethacin (Sigma), 1 µmol/L dexamethasone (Sigma, St. Louis, MO)]. After 48 h, the medium was then replaced with adipogenic maintenance medium (DMEM supplemented with 10% FBS, 1% penicillin–streptomycin, 2 mM glutamine and 10 µg/mL insulin). Cells were cultured continuously for 3 weeks with a medium change two or three times weekly. The cells containing drops of fat were identified by staining the culture with 3.75% Oil Red.

Osteogenic differentiation assay of BMSCs

Passage 5 BMSCs were cultured in 6-well plates in complete culture medium. On the following day, when the cells reached 60% confluence, the medium was replaced with osteo-inductive medium (DMEM supplemented with 10% FBS, 1% penicillin–streptomycin, 2 mM glutamine, 0.2 mM/L ascorbate (Sigma) and 10 mM/L-glycerophosphate (Sigma), and 0.1 µmol/L (dexamethasone), which was changed two to three times a week. The cells were cultured continuously for up to 3 weeks. Calcium containing precipitates were visualised by staining with von Kossa staining.

Phenotypic characterisation of BMSCs

Detection of cell-surface antigen was performed using a BD Biosciences FACS machine as described previously (Prockop *et al.*, 2008). BMSCs were harvested by trypsinisation, washed in phosphate-buffered saline (PBS), and stained by fluorescein isothio-cyanate (FITC) or phyco-erythrin (PE)-coupled antibodies against CD117 (0.5 mg/mL; BioLegend, San Diego, CA), CD44 (0.5 mg/mL; Biolegend) and SCA-1 (0.2 mg/mL; Biolegend), CD29 (0.2 mg/mL; Biolegend) CD34 (0.2 mg/mL; Biolegend). This analysis was completed at passage 5, which was concurrent with the time MSCs were used in the *in vivo* model of acute asthma.

Asthmatic mouse model

The asthmatic mouse model was produced as previously described (Hamelmann *et al.*, 1997). Briefly, sensitisation to OVA (Grade V; Sigma–Aldrich Inc., St. Louis, USA) was conducted with two i.p. injections of 80 µg OVA emulsified in 100 µL of alum hydroxide (Grade V; Sigma) in a total volume of 200 µL. The injections were given at an interval of 14 days. Aerosol challenges were conducted for 30 min on Days 24–26 with 1% OVA in PBS using an ultrasonic nebuliser (air-compressing nebuliser; Jiangsu Yuyue Medical Equipment Inc.).

Experimental designs

Groups of mice (ten mice per group) receiving the following treatments were divided into four groups: (1) sensitisation and challenge to PBS plus airway injection with PBS (PBS group); (2) sensitisation and challenge with OVA plus airway transplantation with BMSCs (BMSCs treatment group); (3) sensitisation and challenge with PBS plus airway transplantation with BMSCs (BMSCs control group); (4) sensitisation and airway challenge with OVA plus airway injection with PBS (asthmatic group; Figure 1).

Intratracheal transplantation of BMSCs

BMSCs were harvested by trypsinisation and washed with PBS as described above, and a cell suspension of 17,000 cells/µL was prepared in PBS. The cell suspension was kept on ice until the BALB/c mice were anaesthetised with pentobarbital sodium (50 mg/kg i.p.; Sigma). Under sterile conditions, the trachea was exposed through a midline incision, and 30 µL PBS containing 5×10^5 BMSCs was injected into the trachea with a 1 mL syringe and a 0.5-in. 23-gauge needle. To aid in the distribution of BMSCs evenly to distal airspaces, the

intratracheal injection was made during a deep inspiration after compression of the thorax. Control animals received an equal volume of PBS. Mice were sacrificed and the lungs were assessed for histology and serum, and bronchoalveolar lavage fluid (BALF) was detected for cytokines at 28 days.

Measurement of airway responsiveness

At 27 days, airway responsiveness (AR) was measured in unrestrained animals by barometric plethysmography using whole body plethysmography (WBP; Buxco) as described previously (Hamelmann *et al.*, 1997). Mice were placed in the main chamber, and baseline readings were taken and averaged for 3 min. Aerosolised PBS, or different concentrations of methacholine (MCh; 6, 12, 25 and 50 g/L; Sigma–Aldrich) were nebulised through an inlet of the main chamber for 3 min. Readings were taken and Penh was used as the measure of airway responsiveness. Airway reactivity was expressed as a fold increase for each concentration of MCh (Penh_{MCh}) compared with Penh values after a PBS challenge (Penh_{PBS}).

Bronchoalveolar lavage

At 28 days, mice were anaesthetised and blood samples were collected by cardiac punctures. BALF was collected by cannulating the trachea and lavaging the lungs three times with 0.3 mL sterile PBS. The aliquots were pooled and the volume was measured. The total cell number from each animal was determined. After being centrifuged at 1,000g for 5 min, the BALF supernatant was frozen for subsequent cytokine assays, and the cell pellet was resuspended for cytocentrifugation on microscope slides. Differential cell counts were performed on slides stained with Diff-Quik (Dade Behring, Duding, Switzerland). At least 400 cells on each preparation were counted for quantitative analysis.

Determination of regulatory $\text{CD4}^+\text{CD25}^+$ T lymphocytes

Multicolor flow cytometric analysis was used to determine the frequencies of $\text{CD4}^+\text{CD25}^+$ T in pulmonary lymph node. Pulmonary lymph node were dissociated and red blood cells were lysed. The cells were stained with fluorescently labelled anti-CD25-APC (0.2 mg/mL; BD Biosciences, San Jose, CA), anti-CD4-FITC (0.5 mg/mL; Bioscience), anti-Foxp3 0.2 mg/mL; Bioscience); isotype-matched antibodies were used as controls for the samples.

Measurement of cytokines

After assessment of AR, BALF and serum from four groups of mouse were collected and analysed with commercially

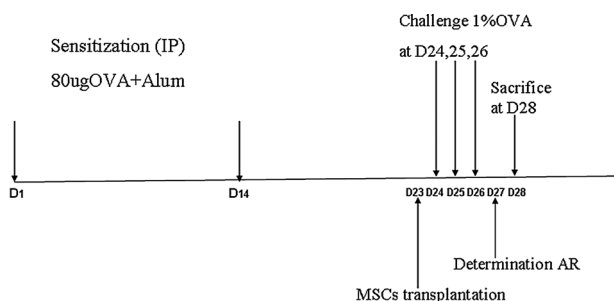


Figure 1 Experimental protocol. Sensitisation to OVA on Days 1 and 14 was by i.p. injection of 80 µg OVA emulsified in 100 µL of alum hydroxide. On Day 23, BMSCs were transplanted intratracheally in asthmatic mice model. One percent of OVA aerosol challenges were given for 30 min on Days 24–26. On Day 27, airway responsiveness (AR) was measured and mice were killed on Day 28.

available Quantikine kits, including interleukin (IL)-4, IL-13, IL-10, IL-12, IFN- γ , TNF- α and TGF- β (PDGF; R&D Systems, Minneapolis, Minn).

Morphometric analysis

After collection of BALF, left lung tissue was perfused with 0.1 mL 4% paraformaldehyde formalin, removed, and fixed in 4% paraformaldehyde formalin. The fixed lung tissue was then sectioned, and stained with periodic-acid Schiff (PAS) and haematoxylin for counting mucin-secreting cells and the identification of eosinophils, respectively. Lung sections stained with HE were assigned a unit value for peri-bronchiolar and peri-vascular areas. The numerical scores for each view field were determined as follows (Ford *et al.*, 2001): 0 = normal; 1 = few cells; 2 = a ring of inflammatory cells with one cell layer deep; 3 = a ring of inflammatory cells with a depth of two to four cells; 4 = a ring of inflammatory cells

with the depth of more than four cells. In sections stained with PAS, medium-sized airways were assessed. The numerical scores for the abundance of PAS-positive goblet cells in each airway were determined as follows: 0: <5%; 1: 5–25%; 2: 25–50%; 3: 50–75%; 4: >75%. Two sections per animal and 10 fields per section were selected for quantitative analysis.

Statistical analysis

Statistical analysis was performed with the statistical SPSS 15.0 software (SPSS Inc., Chicago, IL, USA). Data were expressed as medians and interquartile ranges. The non-parametric Mann–Whitney rank-sum test was used for comparing the median values of eosinophils in BALF between asthmatic group and BMSCs treatment group. Morphometry and cytokine data were compared statistically among the groups by using Kruskal–Wallis test followed by Nemenyi

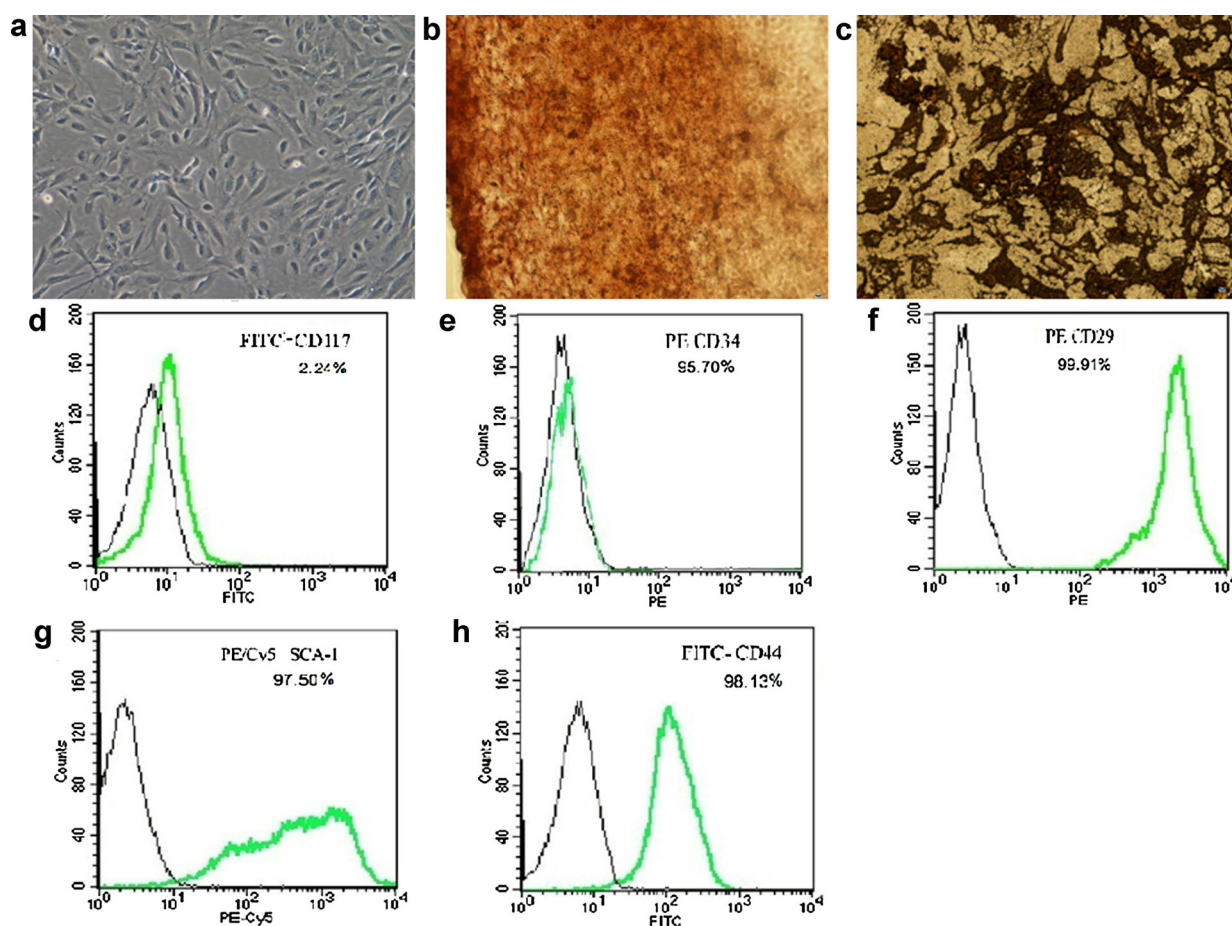


Figure 2 Differentiation assay of BMSCs. (a) Spindle-shaped fibroblast-like appearance of BMSCs. (b) Oil Red staining, showing differentiation of BMSCs into adipocytes (red). (c) von Kossa staining, showing differentiation of BMSCs into osteoblasts. Flow cytometric analysis shows that the cells did not express the cell surface markers CD117, CD34, but expressed the cell surface markers CD29, Sca-1 and CD44, indicating cells isolated from bone marrow had phenotypic features of BMSCs (d–h).

multiple comparison procedure. For airway reactivity, repeated measures of variance analysis were used followed by one-way ANOVA for different concentrations. Statistical significance was defined as $P < 0.05$.

Results

BMSCs have multipotentiality

Using a differentiation assay on BMSCs to identify whether BMSCs have multipotentiality, it was found that after differentiation, morphologically the differentiated cells had a spindle shaped fibroblast-like appearance (Figure 2a). MSCs retained their abilities to form adipocytes (Figure 2b) and osteoblasts (Figure 2c) at passage 5. Flow cytometric showed that the cells did not express the cell surface markers CD117 and CD34; however, they expressed the cell surface markers CD29, Sca-1 and CD44, indicating cells isolated

from bone marrow had phenotypic features of BMSCs (Figures 2d–2h).

BMSCs engraftment significantly reduces the number of inflammatory cells in BALF in asthmatic mice

To determine the effect of BMSCs on inflammation in asthmatic mice, inflammatory cells in BALF were stained and counted. There were no obvious inflammatory cells in the PBS group and BMSCs control group (Figure 3a), but many inflammatory cells in asthmatic group (Figure 3b). However, BMSCs engraftment in asthmatic mice significantly decreased the number of eosinophils by approximately eightfold compared to the asthmatic group ($P < 0.01$) in BALF (Figures 3c and 3d). Eosinophils were identified by eosinophilic coarse particles in the cytoplasm, and the nucleus was usually two lobes, whereas the nuclei of mononuclear cells were large and irregular, the chromatin

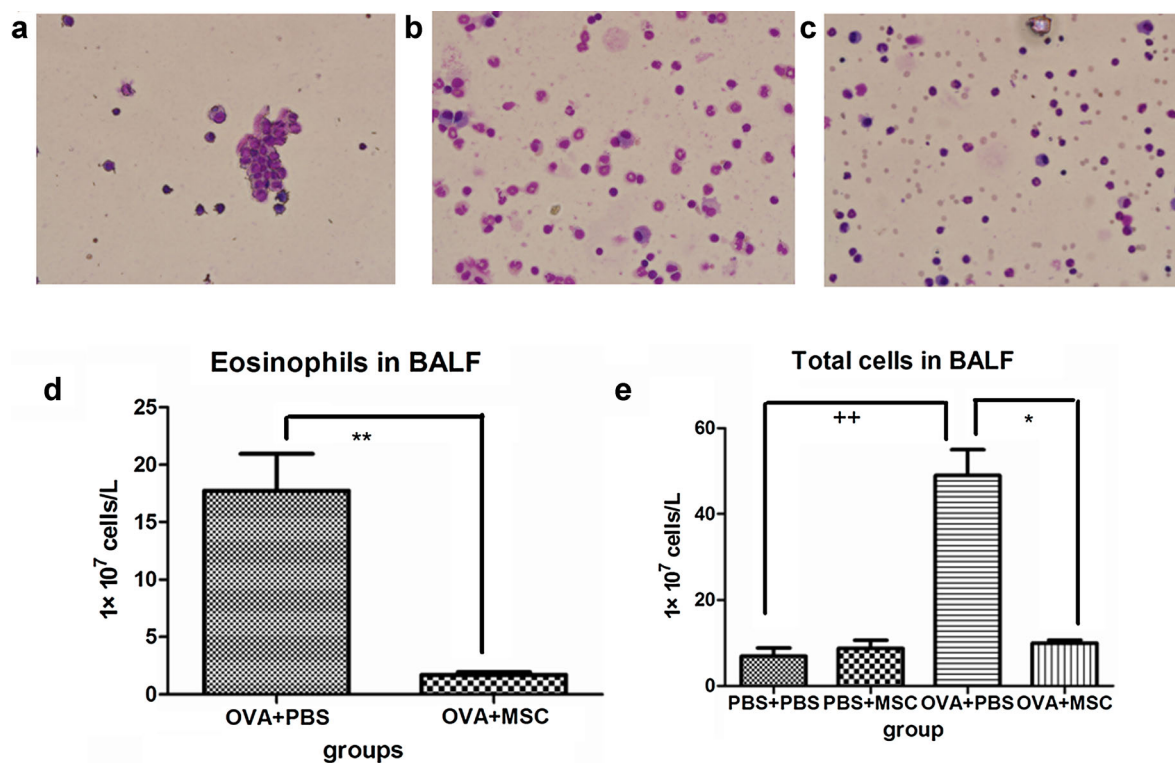


Figure 3 BMSCs engraftment significantly inhibited OVA-induced BALF eosinophils in asthmatic mice. (a) There were no obvious inflammatory cells in BALF in the PBS group. (b) In asthmatic mice, infiltration inflammatory cells, including eosinophils and mononuclear cells, was found in BALF (Diff-Quik staining; magnification 200×). (c) BMSCs engraftment significantly reduced the number of eosinophils and mononuclear cells in BALF (Diff-Quik staining; magnification 200×). (d and e) Quantitative analysis of the number of inflammatory cells in BALF after BMSCs engraftment. The non-parametric Mann–Whitney rank-sum test was used for comparing the median values of eosinophils in BALF between the BMSCs treatment group and asthma group; whereas the total cells in BALF were compared statistically among the groups by using Kruskal–Wallis test followed by Nemenyi multiple comparison procedure. (d) eosinophils in BALF. (e) The total cells in BALF were represented as medians and interquartile ranges. * $P < 0.05$; ** $P < 0.01$ for the comparison between BMSCs treatment group and asthma group. + $P < 0.05$; ++ $P < 0.01$ for the comparison among groups of asthma group, PBS group and BMSCs control group.

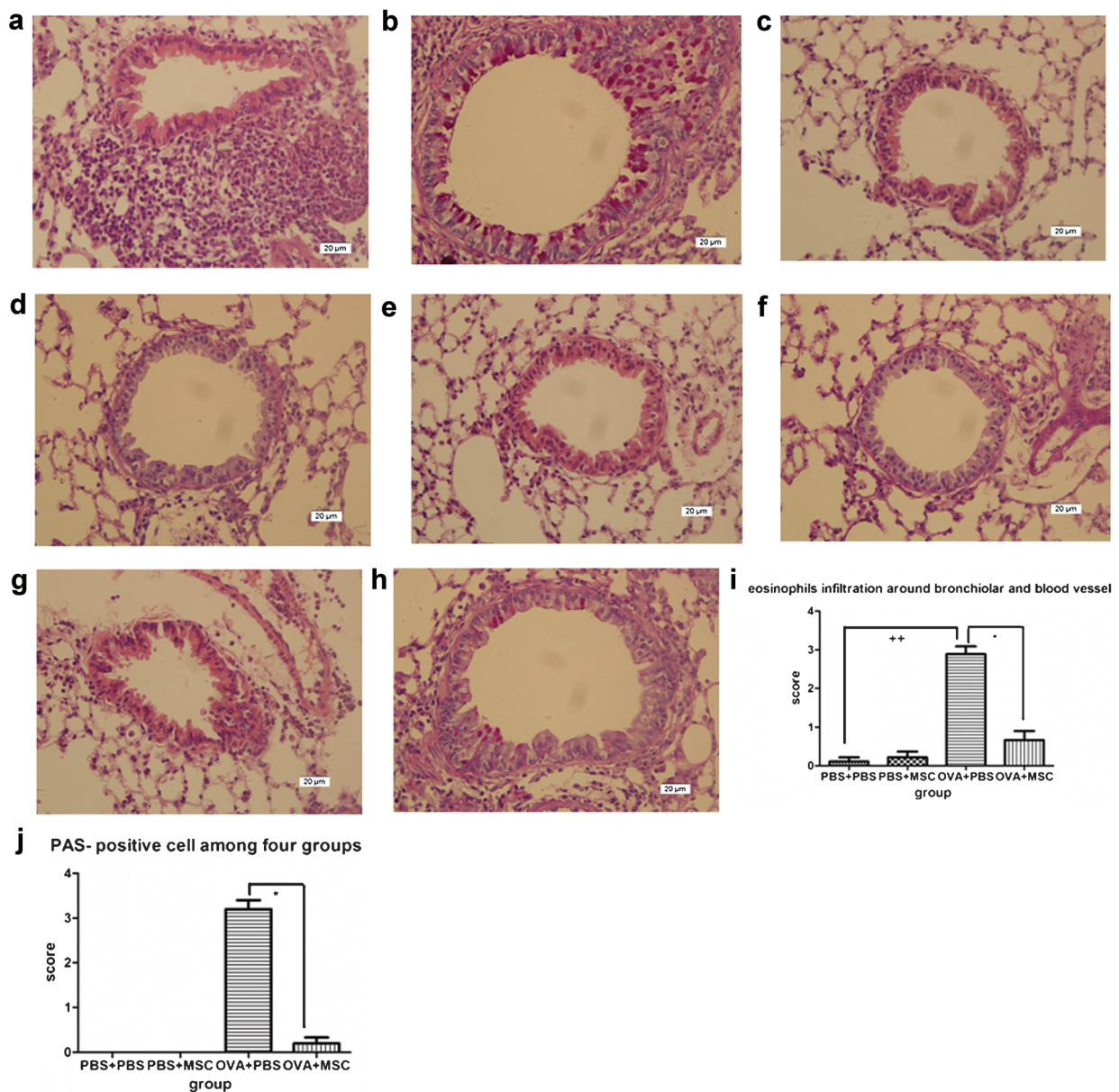


Figure 4 Representative histological staining in the airway from PBS mice and OVA-challenged mice with or without BMSCs transplantation. (a) In the asthmatic group, infiltration of inflammatory cells was observed, especially eosinophils, around the airway and blood vessels. (b) Goblet cell hyperplasia was found in the asthmatic group. (c–f) HE and PAS staining showed no obvious inflammatory cell infiltration and goblet cell hyperplasia in the PBS group (c and d) and BMSCs control group (e and f). (g) Treatment of BMSCs transplantation decreased the infiltration of eosinophils around the airway and blood vessels. (h) Treatment of BMSCs transplantation significantly reduced the number and intensity of PAS-positive cells. (i and j) Quantitative analysis of eosinophil infiltration and goblet cell hyperplasia in the mice with or without treatment of BMSCs engraftment. This treatment markedly inhibited eosinophil infiltration around vessels and in the airways and goblet cell hyperplasia in asthmatic mice. Eosinophils infiltration and goblet cell hyperplasia were compared statistically among the groups using a Kruskal–Wallis test followed by Nemenyi comparison procedure. * $P < 0.05$; ** $P < 0.01$ for the comparison between groups of BMSCs treatment group and asthma group. + $P < 0.05$; ++ $P < 0.01$ for the comparison among groups of asthma group, PBS group and BMSCs control group. HE staining (a, c, e and g) and PAS (b, d, f and h) staining. Magnification 400 $\times$.

pattern was loosely distributed with or without nucleoli, and the cytoplasm appeared as grey-blue containing vacuole and Azure particles. The total cells in the BMSCs treatment group was four times lower than that in asthmatic group (Figure 3e, $P < 0.01$). The results indicate that BMSCs engraftment prohibited infiltration of inflammatory cells in asthmatic mice.

BMSCs engraftment significantly reduces the infiltration of inflammatory cells and goblet cell hyperplasia in the airway in OVA-challenged mouse

No obvious infiltration of inflammatory cells was found in the PBS group (Figure 4c) and the BMSCs control group (Figure 4e), but a great number of eosinophils around the peribronchiolar and blood vessel was seen in the asthmatic group (Figure 4a). Furthermore, BMSCs administration reduced inflammatory cells in asthmatic mice (Figure 4g). Concurrently, PAS-stained goblet cell hyperplasia, as demonstrated by the increased number and size of goblet cells, also occurred within the respiratory epithelium in the asthmatic group (Figure 4b). However, this hyperplasia was not found in the airways both in the PBS group (Figure 4d) and the BMSCs control group (Figure 4f).

Interestingly, BMSCs transplantation induced a significant reduction the number of eosinophils in the airway (Figure 4i, $P < 0.05$) and goblet cell hyperplasia in asthmatic mouse (Figure 4h–4j).

BMSCs engraftment reduces airway hyperresponsiveness in asthmatic mice

To identify the effect of MSCs on lung function after engraftment, AR was measured. Penh in four groups were increased with increasing concentrations of MCh (Figure 5). Penh in asthmatic mice at 12.5–50 g/L were approximately twofold higher compared with that in the PBS group and BMSCs control group (Figure 5, $P < 0.05$). In contrast, BMSCs treatment significantly decreased at different concentrations from 12.5 to 50 g/L in response to MCh (Figure 5) compared to the asthmatic group. The findings suggest that BMSCs had a significant effect on airway reactivity in asthmatic mice.

BMSCs engraftment increases the frequencies of CD4⁺CD25⁺ regulatory T cells in asthmatic mice

To study the mechanism of BMSCs effect, CD4⁺CD25⁺ regulatory T cells were explored; their frequencies in pulmonary lymph node in asthmatic group was significantly lower than that in PBS group and BMSCs control groups ($P < 0.05$). In addition, the frequencies of CD4⁺CD25⁺ regulatory T cells in pulmonary lymph node was markedly increased by administrating BMSCs in asthma ($P < 0.01$; Figure 6).

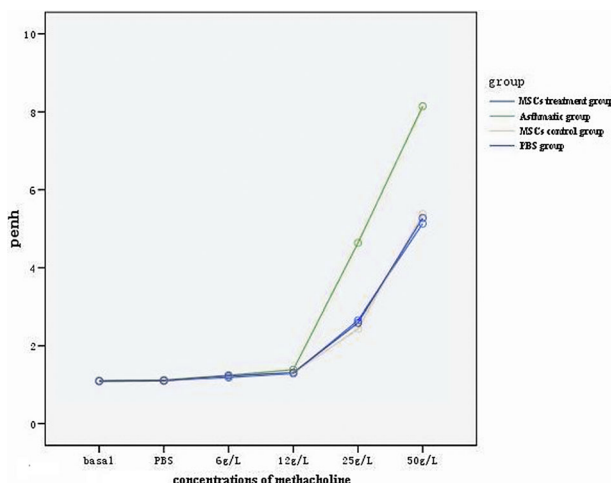


Figure 5 Effect of BMSCs engraftment on airway hyperresponsiveness in asthmatic mice. Airway hyperresponsiveness (AHR) of basal values or the response to nebulised PBS and to four concentrations of methacholine (6, 12, 25 and 50 g/L) were measured with whole-body plethysmography. Nine mice were used in the BMSCs control group and 10 mice in the other groups. Airway reactivity to methacholine challenge in asthmatic group was significantly higher than in the PBS group and BMSCs control group at three concentrations of methacholine (12, 25 and 50 g/L). In contrast, BMSCs engraftment significantly decreased responsiveness to methacholine compared to the asthmatic group ($P < 0.05$).

Effect of BMSCs engraftment on levels of IL-10 and IL-12 in BALF and serum in asthmatic mice

Cytokine levels were measured to follow the effect of BMSC engraftment in asthmatic mice. ELISA analysis showed no difference on the level of IL-10 and IL-12 in the PBS group

TREG in lymphoid node in different group

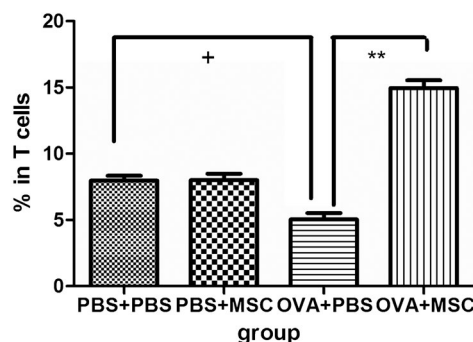


Figure 6 To study the mechanism of BMSCs effect, CD4⁺CD25⁺ regulatory T cells were determined. The frequency of CD4⁺CD25⁺ regulatory T cells in pulmonary lymph node in asthmatic group was significantly lower than in the PBS group and the BMSCs control groups ($P < 0.05$). The frequency of CD4⁺CD25⁺ regulatory T cells in pulmonary lymph node was increased markedly by administrating BMSCs in asthma ($P < 0.01$).

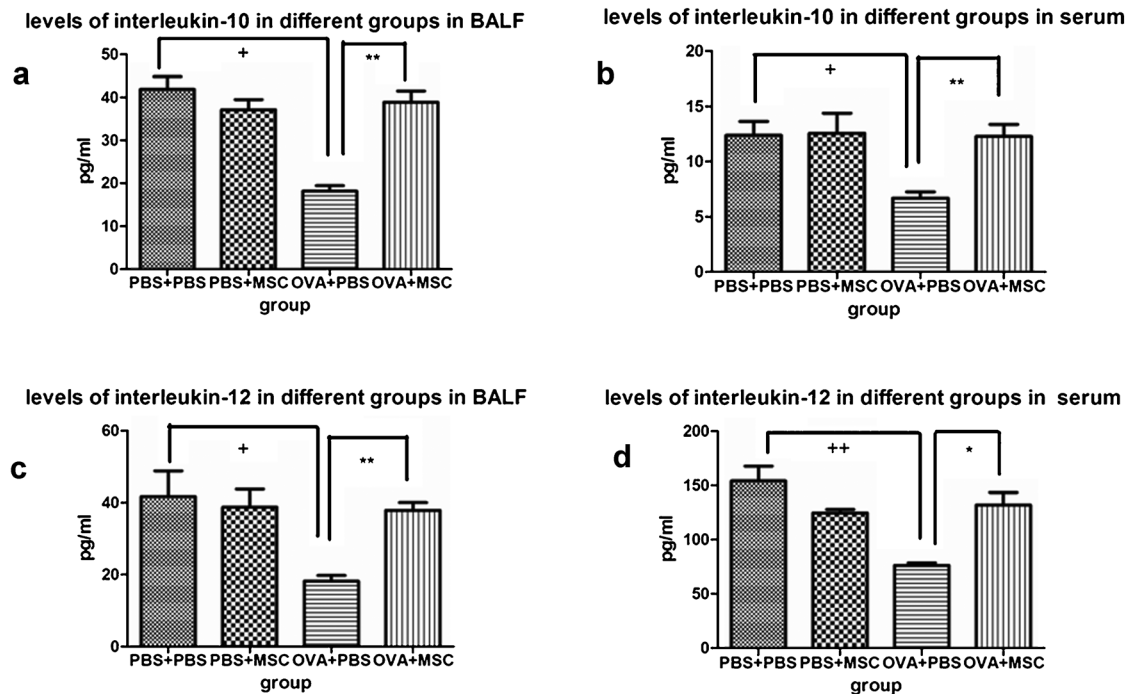


Figure 7 Effect of BMSCs engraftment on levels of IL-10 and IL-12 in BALF and serum in asthmatic mice. On Day 28, a variety of cytokines among four groups mice were measured by ELISA. Statistical analysis used the Kruskal–Wallis test followed by Nemenyi multiple comparison procedure. Data for (a) IL-10 in BALF (b) IL-10 in serum (c) IL-12 in BALF and (d) IL-12 in serum were represented as medians and interquartile ranges. OVA challenge induced a significant reduction in the levels of IL-10 and IL-12 in BALF and serum in asthmatic group. However, BMSC engraftment significantly increased both IL-10 and IL-12 levels in BALF and serum. * $P < 0.05$; ** $P < 0.01$ for the comparison between groups of BMSCs treatment group and asthma group. + $P < 0.05$; ++ $P < 0.01$ for the comparison among the asthmatic, PBS and BMSC control groups.

and the BMSCs control group in BALF and serum (Figures 7a–7d). However, OVA challenge induced a significant reduction in the levels of IL-10 and IL-12 in BALF and serum in asthmatic group (Figures 7a–7d, $P < 0.05$). In contrast, BMSCs engraftment induced significantly higher levels of IL-10 and IL-12 in BALF and serum than those in the asthmatic group (Figures 7a–7d, $P < 0.05$). However, BMSCs engraftment did not affect the levels of IL-13, IL-4, TNF- α , TGF- β and IFN- γ in BALF and serum (Figures 8a–8j).

Discussion

OVA challenge induced an infiltration of inflammatory cells in the airway and BALF as well as goblet cell metaplasia in the airway of asthmatic mice. BMSCs administration led to a significant histological and functional improvement in these mice. These findings indicate that BMSCs inhibited recruitment of eosinophils and mononuclear cells into the airway and BALF, and reduced airway responsiveness in asthmatic mice, which is consistent with data on MSCs (Cho and Roh, 2010; Nemeth et al., 2010).

I.v. delivery of BMSCs in disease models has been commonly used. However, intravascular administration of MSCs can cause death in small laboratory animals due to pulmonary embolism, and the relatively large cell size of MSCs results in occlusion in pulmonary capillaries (Barbash et al., 2003; Furlani et al., 2009). Local delivery of MSCs, for instance, the intratracheal delivery of MSCs in acute lung injury (ALI) mice model and the left ventricular cavity delivery of MSCs in myocardial infarction model, has been shown to be a more efficient method for cell engraftment (Wang et al., 2005; Walczak et al., 2008). Here we employed an intratracheal route to deliver BMSCs in asthmatic mice, the data indicating that local delivery of BMSCs does indeed improve lung function and prevents airway inflammation due to asthma.

The mechanisms underlying the beneficial effect of BMSCs in asthma are unclear. Administration of MSCs can ameliorate severity of ALI and fibrosis (Anjos-Afonso et al., 2004; Yamada et al., 2004; Wang et al., 2005; Iso et al., 2007) and modulation pro-inflammatory and anti-inflammatory cytokine is considered as the main beneficial effect of MSCs. Since asthma is characterised as chronic

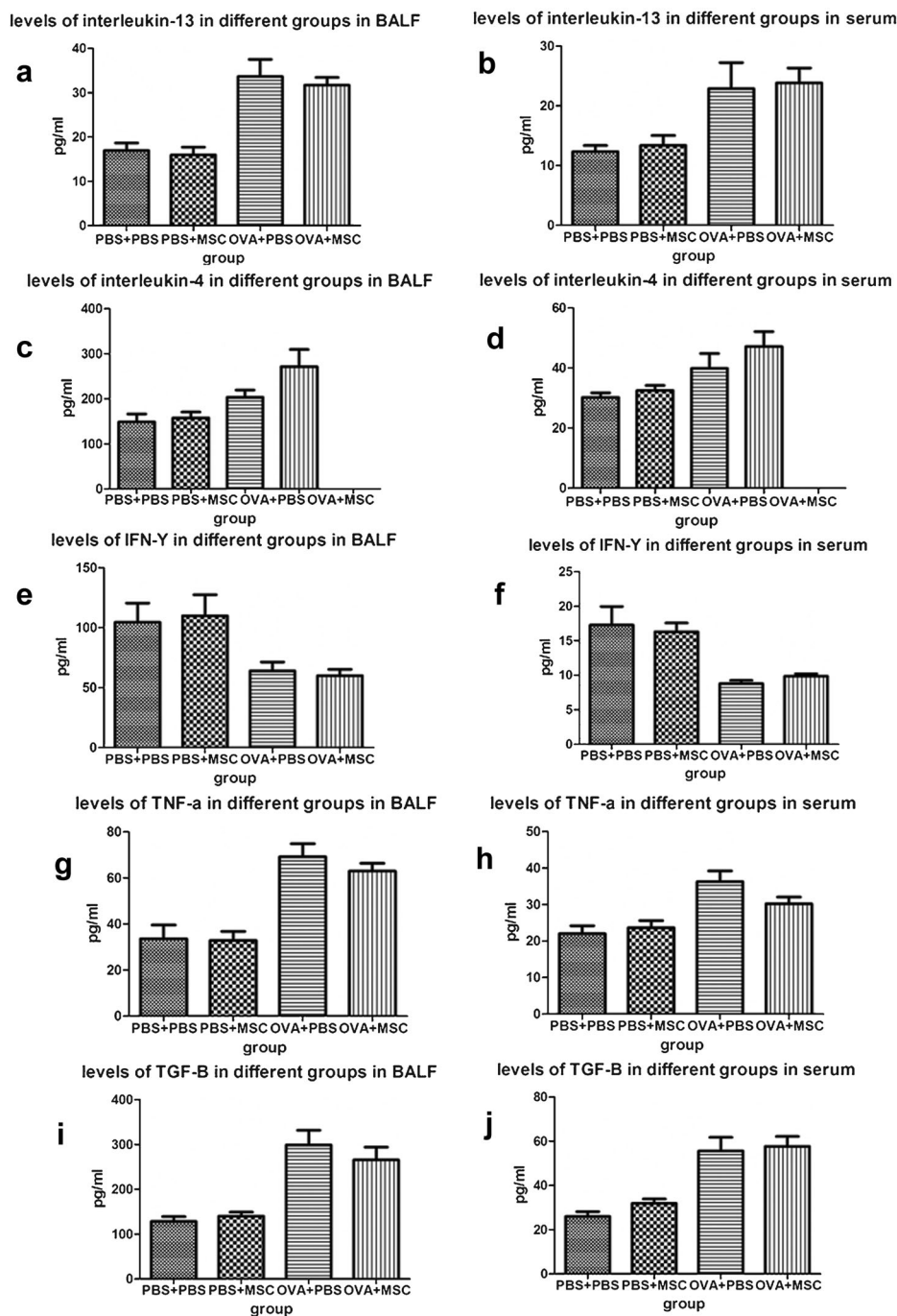


Figure 8 Effects of BMSCs engraftment on levels of IL-4, IL-13, TNF- α , TGF- β and IFN- γ in both BALF and serum in asthmatic mice. BMSCs engraftment did not affect the levels of IL-13 (a and b), IL-4 (c and d), IFN- γ (e and f), TNF- α (g and h) and TGF- β (i and j) in BALF and serum. * $P < 0.05$; ** $P < 0.01$ for the comparison between groups of BMSCs treatment group and asthma group. + $P < 0.05$; ++ $P < 0.01$ for the comparison among groups of asthmatic, PBS and BMSC control groups.

airway inflammation and unbalance between TH1- and TH2-derived cytokines. We thus propose that BMSCs-driven immunomodulation contributes to attenuation of airway inflammation in asthma, consequently, improving lung function, and quality of life.

Regulatory T cells are a unique T cell population with strong immunosuppressive properties. CD4⁺CD25⁺ regulatory T cells were impaired quantitatively and functionally in asthma (Hartl et al., 2007). CD4⁺CD25⁺ T cells also play a protective role in suppressing airway eosinophilic

inflammation and the development of airway hyperreactivity in asthma. Moreover, anti-inflammatory effect of CD4⁺CD25⁺ T cells is dependent on the presence of IL-10 (Akbari et al., 2002; Jaffar et al., 2004). We detected Tregs in pulmonary lymph node among the four groups, which indicates that the ratio of Tregs in pulmonary lymph node was lower in asthmatic model group than that in the PBS and BMSCs control groups, which was similar to previous studies that indicate that BMSCs preferentially activate CD4⁺CD25⁺ T cell subsets, which are the main underlying mechanisms for immunosuppressive activity of MSCs (Maccario et al., 2005; Selmani et al., 2008). Augello et al. (2007) showed allogeneic BMSCs in murine rheumatoid arthritis model prevents severe irreversible damage by inducing production of antigen-specific Tregs. However, the effect of BMSCs on Tregs in asthma is still unknown. Our ratio of Tregs in pulmonary lymph nodes was lower in asthmatic model group than that in the PBS group, whereas BMSCs engraftment significantly increased the ratio of Tregs in pulmonary lymph node in asthma.

IL-10 is a potent endogenous anti-inflammatory molecule that decreases inflammation in the lung partly via inhibition of TNF- α and IL-1 beta in ALI mice (Shanley et al., 2000; Spight et al., 2005). Furthermore, Aggarwal and Pittenger (2005) showed that co-culture of MSCs with dendritic cells could enhance dendritic cell IL-10 secretion. IL-10 is involved in the suppressive effect of Treg cells in inflammatory diseases (Rubtsov et al., 2008). IL-10-deficient Tregs are unable to completely suppress them in autoimmune gastritis and colitis (Asseman et al., 1999; Suri-Payer and Cantor, 2001), whereas IL-10 partly contribute to suppression of autoimmune gastritis and colitis induced in mice by Treg depletion. However, the relationship between IL-10 and Treg in asthma was seldom involved. In our BMSC engraftment, an increase in the ratio of Tregs in pulmonary lymph node and IL-10 in asthma was seen. Thus, we proposed that MSCs exert anti-inflammatory effects by upregulating Treg and are partly involved in increasing the protective cytokine IL-10 in asthma.

IL-12 also has anti-inflammatory activity in the lung for inhibition of airway hyperresponsiveness (AHR) and eosinophil infiltration (Naseer et al., 1997; Matsuse et al., 2003; Onari et al., 2009). Our BMSCs administration increased the level of IL-12 in both serum and also BALF in asthmatic mice, consistent with other studies (Rojas et al., 2005). However, the mechanism underlying BMSCs upregulation of IL-12 in asthma remains unclear.

In conclusion, the present study demonstrates that intrapulmonary treatment with BMSCs in experimental OVA-induced asthma provides a significantly functional and histological advantage in reduction of airway response to MCh, and decreases inflammation response in the airway. This protection might be mediated by upregulating Treg and

partly involves increasing protective cytokine IL-10 in asthma.

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