

Original Article

Heme oxygenase 1 delivery via adipose derived stem cells reduces airway inflammation in mice asthma model

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Abstract: Objectives: Heme oxygenase 1 (HO-1) is an enzyme induced by stress with effects to protect cells, and recently reported to have an important role in anti-inflammation. To investigate whether HO-1 delivery via adipose tissue-derived stem cells (ADSCs) can be over expressed in lung tissue and inhibit the asthma characteristic feature in asthmatic model of mice. We examined the expression of HO-1, infiltration of inflammatory cells, airway hyper-responsiveness (AHR) and cytokine levels in BALF in asthmatic model of mice. Methods: HO-1 cDNA was prepared via RT-PCR method to produce lentiviral vector with HO-1 gene, HO-1 gene was transfected into cultured ADSCs using a lentiviral vector. Mice were sensitized and challenged with ovalbumin (OVA). 0.1 ml (2×10^6) ADSCs with HO-1 gene, ADSCs or saline was injected into tail vein of mice 24 h prior to challenge with 3% OVA for 7 days. Characteristic features of asthma were measured 24 h after the last challenge. Results: HO-1 can be over expressed in lung tissue. AHR was significantly decreased in ADSCs/HO-1 group compared with that in ADSCs and OVA group. Inflammatory cells in lung tissue and bronchoalveolar lavage fluid (BALF) were significantly decreased in ADSCs/HO-1 group compared with ADSCs and OVA group. Cytokines IL-4, IL-5, IL-13 were decreased and IL-10, IFN- γ were increased in ADSCs/HO-1 group compared with ADSCs and OVA group. Conclusions: HO-1 delivery via ADSCs can be over expressed and exert its protective effect on asthma through a mechanism mediated by decreasing IL-4, IL-5, IL-13 and increasing IL-10, IFN- γ .

Keywords: Asthma, ADSCs, heme oxygenase 1, gene therapy, mouse

Introduction

Asthma is a kind of chronic disease, characteristic with airways inflammation and AHR [1]. At present, the main therapies for asthma are control of symptom via corticosteroids or specific inflammatory mediator antagonists [2]. However, the effects of anti-inflammatory drugs are not long-lasting and there are significant side effects caused by those drugs in some cases.

Some studies have recently demonstrated that administration of mesenchymal stem cells (MSCs) can reduce the allergic inflammation of airway in mouse asthma model [3]. But the effects of anti-inflammation of MSCs are not long-lasting [4], it is the possible reason that

MSC will differentiate into adipocyte, osteocyte, chondrocyte. But, the potential of MSCs as vehicle for exogenous gene therapy is being evaluated with a rising interest about combining gene therapy with cell transplantation [5]. MSCs are the optimal carrier to deliver target genes because they can self-renew, easily expand ex vivo and engraft well. Adipose tissue is abundant, accessible, and easy to obtain from the body. Furthermore ADSCs can be efficiently transduced with retroviral vectors for permanent gene expression [6].

Previously gene therapy was most applied in those incurable diseases (such as cancer and cystic fibrosis), recently it have also focused on other diseases such as asthma and allergic rhinitis [7]. Recently some studies demonstrated

that overexpression of endothelial nitric oxide synthase and IFN- λ 1 (IL-29) can attenuate allergic inflammation of airway and AHR in allergic mouse asthma model [8, 9]. But there are many cytokines involved in pathogenesis of asthma; only the gene of one cytokine was chosen to treat asthma in previous studies, so the effect was not sufficient.

HO is the rate-limiting enzyme that degrades heme into carbon monoxide, ferritin and biliverdin [10]. Recent report show that over expression of HO-1 can attenuate inflammation of airway mediated by TNF- α through down-regulation of TNFR1-dependent oxidative stress [11]. But over expression of HO-1 via induction is not long term or transient. Fortunately the transferred gene can be integrated into the host genome via lentiviral vectors, and thus the protein can be permanently expressed [12].

In the current study, we try to clone the HO-1 gene into lentiviral vectors, choose ADSCs as carrier to deliver HO-1 gene to investigate whether HO-1 delivery via ADSCs exerts effect on asthma in mouse model. We anticipated that HO-1 delivery via ADSCs could be overexpressed in lung tissue, reduced the AHR, decreased the characteristic feature of asthma and restored the balance of Th1/Th2.

Materials and methods

Animals

Female specific pathogen-free BALB/c mice (6-8 weeks of age) were obtained from experimental animal center of Soochow University (Suzhou, China). Mice were housed in an animal facility under standard laboratory conditions of humidity (60-70%), temperature (20-25°C), and provided water and standard chow ad libitum. All experiments including mice were approved by the Institutional Animal Care and Use Committee of Soochow University.

Generation of recombinant lentiviral vectors

The cDNA fragment encoding the mouse HO-1 was cloned from mouse spleen with RT-PCR as previously described with minor modification [13]. The amplified cDNA of HO-1 was inserted into the clone vector pMD18T (TAKARA, Japan) and then into the expression vector pCDH-CMV-MCS-EF1-GFP-Puro (system biosciences, USA) to generate a recombinant plasmid with

HO-1 gene, termed as pCDH-CMV-HO-1, which has been previously described [14]. Lentiviruses were produced by transfection of 293T cells (Cell Bank of the Chinese Academy of Sciences, Shanghai, China) with 20 μ g pCDH-CMV-HO-1, 15 μ g psPAX2 (Addgene, Cambridge, MA, USA) and 6 μ g pMD2.G (Addgene, Cambridge, MA, USA) [15]. Forty-eight hours after transfection, supernatants containing the lentiviral particles were collected and frozen at -80°C until use.

ADSCs infection with lentiviruses

ADSCs for this study were prepared as previously described with minor modification [16]. ADSCs were grown to 80% confluence and infected with lentiviruses with HO-1 at a multiplicity of infection (MOI) of 100. Expression of HO-1 protein in ADSCs was examined by Western Blot.

Sensitization and challenge

The mice were randomly divided into four groups as follows: normal control (NC) group, OVA group, ADSCs with HO-1 (ADSCs/HO-1) group and ADSCs group. There were 8 mice in each protocol group. Mice except NC group were sensitized with an intraperitoneal injection of 20 μ g OVA (Grade V, Sigma, USA) emulsified in 4 mg aluminum hydroxide (Sigma, USA) in 200 μ l saline (pH 7.4) on days 0, 7 and 14, challenged with an aerosol of 3% OVA (Grade II, Sigma, USA) for 30 min using a nebulizer (PARIBOY, Germany) on days 21 to 27. Mice of NC group were sensitized and challenged with saline instead of OVA. For OVA or saline inhalation, mice were placed in a plexiglas inhalation chamber maintained under normoxic and normocapnic conditions. Mice in ADSCs/HO-1 or ADSCs group were injected with 0.1 ml ADSCs encoding HO-1 (2×10^6 cells) or 0.1 ml ADSCs (2×10^6 cells) via tail vein once on day 20. Mice in NC and OVA group were injected with 0.1 ml saline via tail vein once on day 20. Twenty-four hours after the last challenge, the mice were prepared for the examination of AHR, the collection of blood, BALF and lung tissues.

Measurement of airway hyperresponsiveness

AHR was determined twenty-four hours after the last OVA challenge using an AniRes 2005 Lung Function System (Bestlab, 2.0 Beijing, China). BALB/c mice were intraperitoneal inject-

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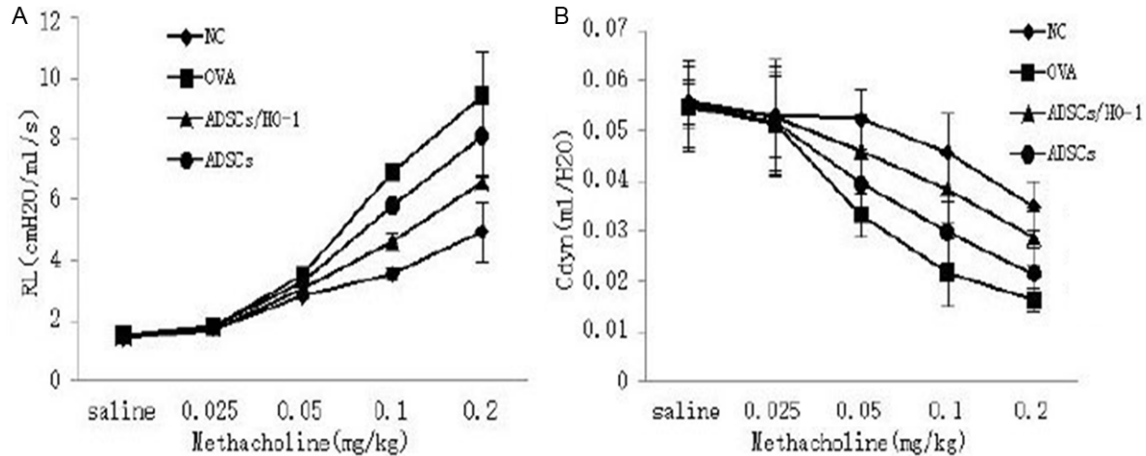


Figure 1. Effect of HO-1 delivery via ADSCs on AHR to Mch. A. RL of mice challenged with gradient doses of Mch. B. Cdyn of mice challenged with gradient doses of Mch. One day after the last OVA challenge, AHR was measured in response to increasing doses of Mch (0-0.2 mg/kg) in conscious mice placed in a whole-body plethysmograph. Mice in NC group were sensitized and challenged with normal saline. Mice in OVA group were immunized and challenged with OVA. Mice in ADSCs/HO-1 and ADSCs groups were immunized and challenged with OVA and treated with ADSCs/HO-1 and ADSCs respectively. Results were expressed as the mean \pm SEM. * $P < 0.01$ as ADSCs/HO-1 group compared with OVA and NC group, † $P < 0.01$ between ADSCs/HO-1 and ADSCs group.

ed with 0.2 ml 10% chloral hydrate to anesthetize, the trachea was surgically exposed, and the mice were placed in a sealed whole-body plethysmograph and then connected with a ventilator controlled by computer via a cannula of 22-gauge vein indwelling needle. The respiratory rate and the time ratio of expiration/inspiration were set at 90/min and 1.5:1, respectively. Saline was injected via tail vein to determine the basic value of resistance of lung (RL) and dynamic compliance (Cdyn). After reaching a stable tracing, methacholine (MCH) was successively injected at a dose of 0.025, 0.05, 0.1 and 0.2 mg/kg body weight at 5-min intervals. After each injection, the mean RL and Cdyn were recorded by the system to reflect the change in airway responsiveness [17].

Collection of bronchoalveolar lavage fluid

Bronchoalveolar lavage (BAL) was performed through an intratracheal tube infusing 3×1 ml aliquots of saline into the right mainstem bronchus while ligating the left main branch of the trachea after assessment of airway function. The recovered fluid (80% of the injected volume) was centrifuged at $1500 \times g$ at 4°C for 10 min and the supernatant was collected to store at -80°C until testing, and cell pellets were resuspended in 0.5 ml PBS for cell counts. Total cells in BALF were counted with a hemacytometer. A differential cell count were con-

ducted at least 200 cells by Wright-Giemsa (Tianhe, Hangzhou, China) staining to identify eosinophils, lymphocyte, neutrophil, and macrophage.

Analyses of lung histology

Left upper lung tissues were fixed with 4% formalin after BALF were collected and embedded in paraffin, and then sectioned (4 μm). Sections were stained with hematoxylin and eosin (H&E, Cellchip Biotechnology Ltd., Beijing, China), and examined under light microscope. Sections were controlled for proximal airways for comparison purposes. Inflammation was scored on a scale from 0 to 4, in blinded fashion by two specialists using an established scoring system [18].

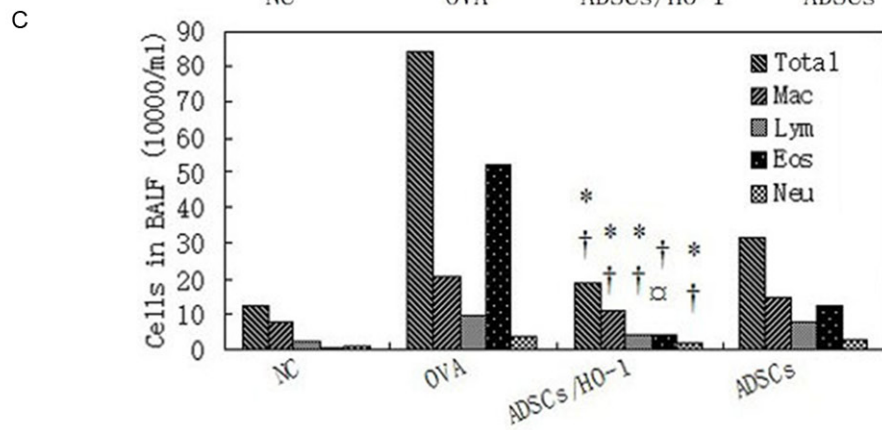
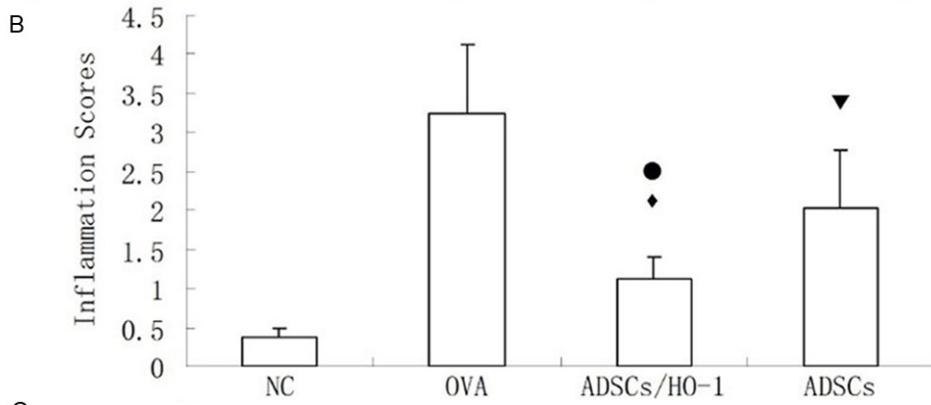
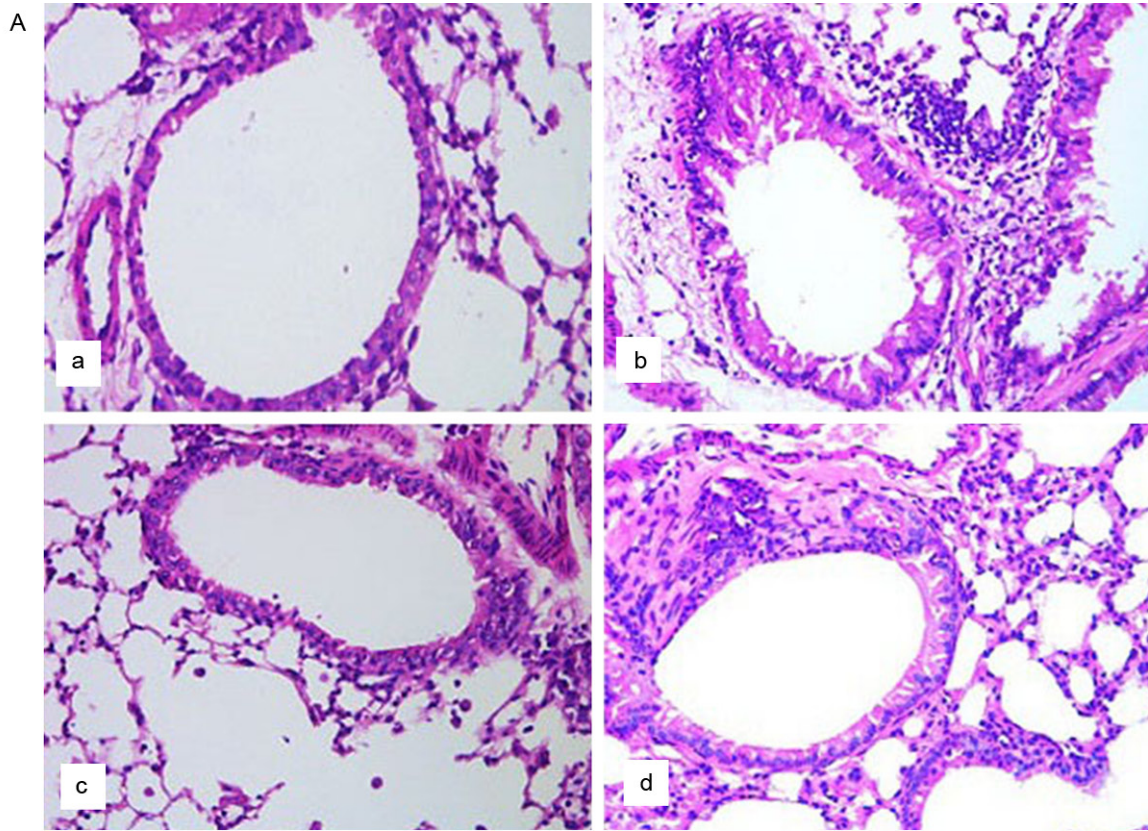
Estimation of cytokines in BALF

The concentration of cytokines including IL-4, IL-5, IL-13, IL-10, and IFN- γ in BALF samples was assessed by ELISAs according to the manufacturer's instructions (Jingmei, Shenzhen, China). The absorbance was read by using the microplate reader at 450 nm. Cytokine concentrations were qualified by comparison to the standard curve.

Immunohistochemical analysis

After paraffin sections were deparaffinized, dehydrated, washed in PBS for 3 min three

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Figure 2. Effect of HO-1 delivery via ADSCs on inflammation in lung. A: Representative H&E staining images of lung sections by H&E staining. (a): NC group, (b): OVA group, (c): ADSCs/HO-1 group, (d): ADSCs group. Examination of lung tissue was performed after the last OVA challenge. Lung tissues were fixed, sectioned at 4 μ m thickness, and stained with H&E solution (magnification \times 400). Section from OVA group displayed inflammatory cell infiltration around bronchial walls. These histological changes were ameliorated in sections from ADSCs/HO-1 or ADSCs groups. B: Total lung inflammation scores. Inflammation score was performed based on the method of Arnaboldi et al, as described in Materials and Methods. Results were expressed as the mean \pm SEM. C: The total and differential cell numbers in the BALF were also determined. The total and differential cell numbers in BALF were significantly higher in the OVA group than in NC group but reduced in ADSCs/HO-1 or ADSCs. Results were expressed as the mean \pm SEM. \bullet $P < 0.05$ as compared with NS group. \blacklozenge $P < 0.01$ as compared with OVA group. \blacktriangledown $P < 0.05$ as compared with ADSCs group. \ast $P < 0.05$ as compared with NC group. \odot $P < 0.01$ as compared with NC group. \dagger $P < 0.01$ as compared with OVA and ADSCs group.

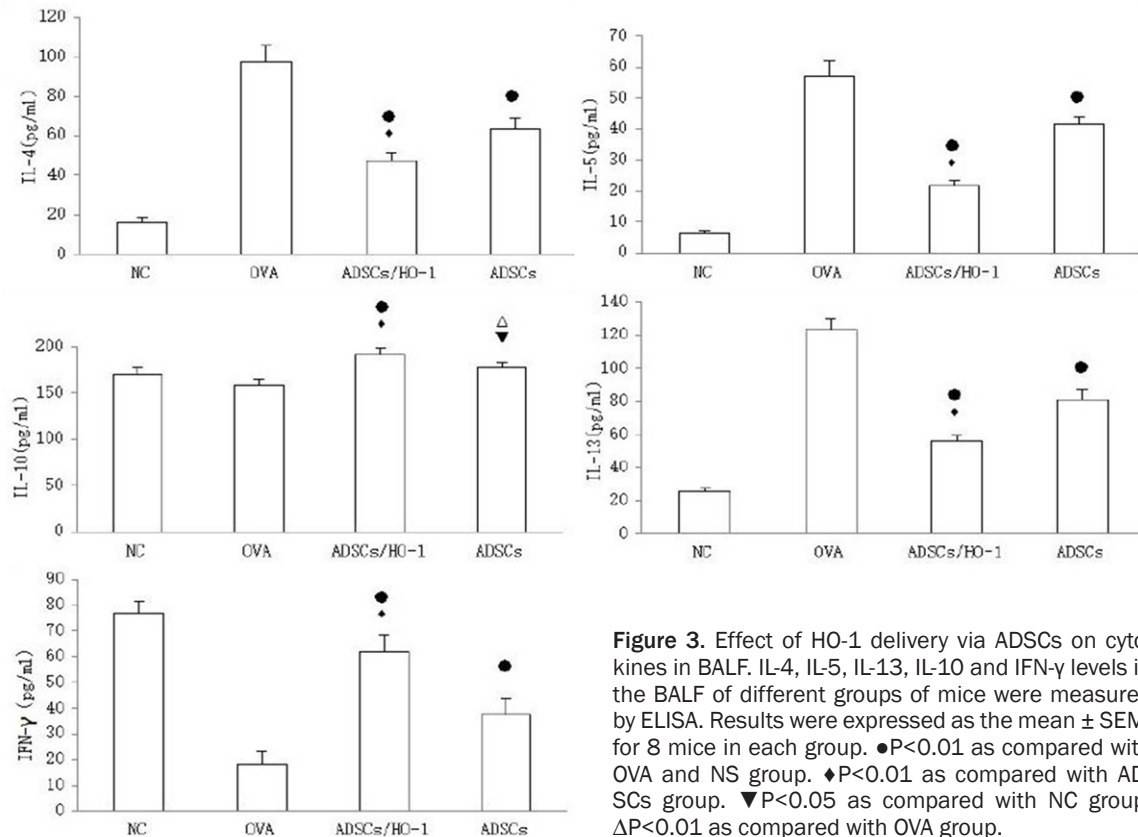


Figure 3. Effect of HO-1 delivery via ADSCs on cytokines in BALF. IL-4, IL-5, IL-13, IL-10 and IFN- γ levels in the BALF of different groups of mice were measured by ELISA. Results were expressed as the mean \pm SEM. for 8 mice in each group. \bullet $P < 0.01$ as compared with OVA and NS group. \blacklozenge $P < 0.01$ as compared with ADSCs group. \blacktriangledown $P < 0.05$ as compared with NC group. Δ $P < 0.01$ as compared with OVA group.

times, each specimen was treated with 3% hydrogen peroxide and preincubated for 10 min at room temperature with 10% goat serum to block non-specific staining, and then incubated with primary anti-HO-1 (1/1000 dilution; Abcam, Cambridge, UK) for 1 hr at 37°C. After removal of primary antibodies, sections were washed and incubated with biotinylated secondary antibody (Zhongshan Golden Bridge Biotech, Beijing, China) at 37°C for 30 min, followed by the avidin-biotin-peroxidase complex (Zhongshan Golden Bridge Biotech, Beijing, China) for 30 min at 37°C. The antibody reaction was visualized using diaminobenzidine

(DAB, Zhongshan Golden Bridge Biotech, Beijing, China) solution of 100 μ l. The sections were counterstained with hematoxylin. All images were captured and analyzed by Image-Pro Plus 6.0 (Media Cybernetics, Silver Spring, USA).

Statistical analysis

The SPSS 17.0 software was used for statistical analysis. Data are expressed as means \pm SEM. Comparisons of means among multiple groups were performed with one-way ANOVA. When significance was indicated, groups were

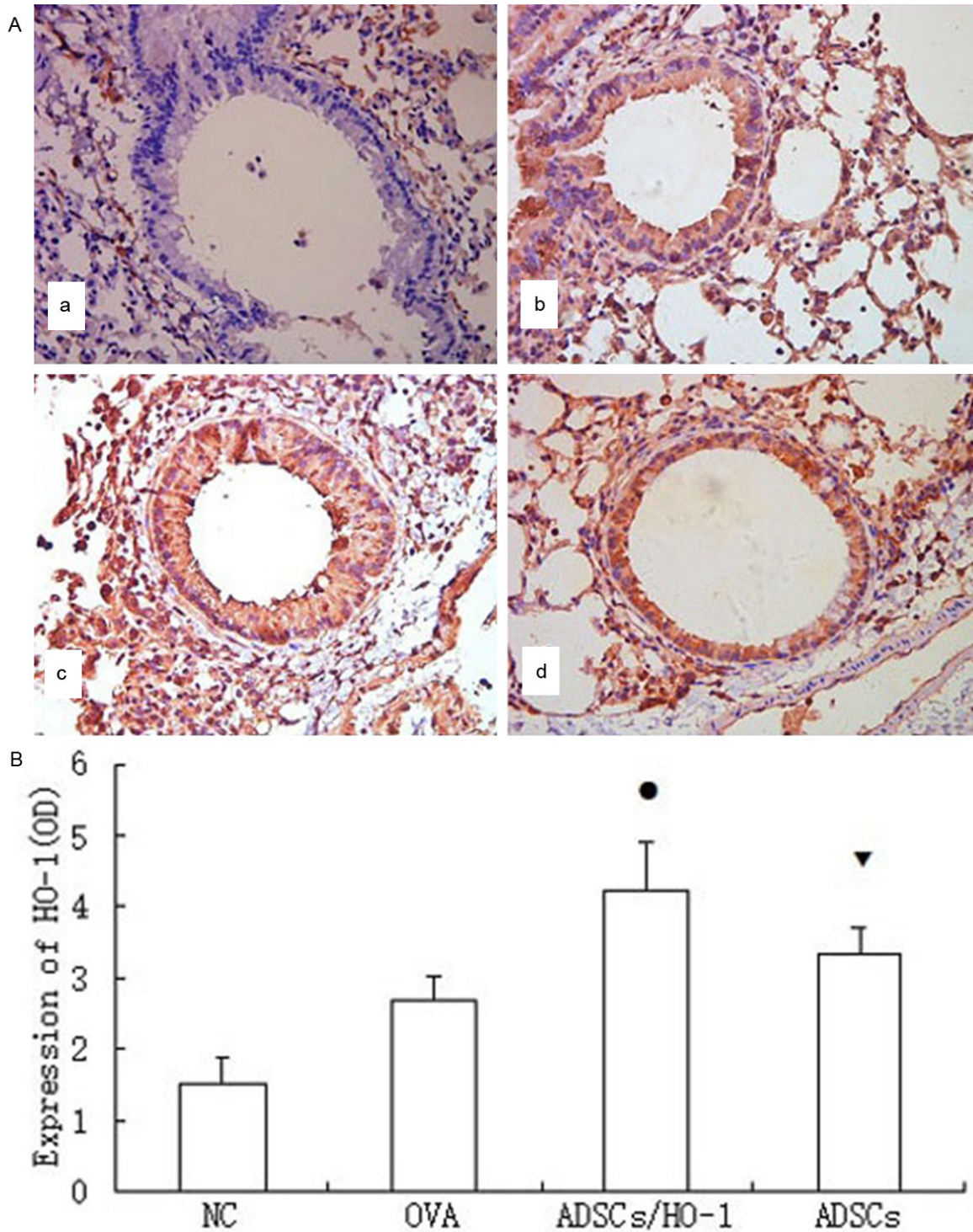


Figure 4. Effect of HO-1 delivery via ADSCs on expression of HO-1 in lung tissue. A: Changes in expression of HO-1 in the lung tissues of female Balb/c mice. (a): NC group, (b): OVA group, (c): ADSCs/HO-1 group, (d): ADSCs group. Representative photomicrographs show staining for HO-1 (magnification $\times 400$). B: OD value of HO-1 expressed in lung tissue captured and analyzed by Image-Pro Plus 6.0. Results are expressed as the mean \pm SEM. ● $P < 0.01$ as compared with OVA and NS group, ▼ $P < 0.05$ as compared with OVA group.

compared using post hoc Dunnett's test. Where significant differences were indicated. Diffe-

rences were considered to be statistically significant when $P < 0.05$.

Results

Effect of HO-1 delivery via ADSCs on AHR

AHR is a characteristic feature of the airway allergic response. In all groups, the RL increased with increasing MCH levels, whereas the Cdyn decreased. We found that MCH caused a dose-dependent increase in RL and a dose-dependent decrease in Cdyn in four groups. When doses of MCH are less than 0.025 mg/kg, no statistical difference was noted in RL and Cdyn among all groups. When doses of MCH are more than 0.05 mg/kg, RL in ADSCs/HO-1 and ADSCs group was significantly lower than that of the OVA group, Cdyn in ADSCs/HO-1 and ADSCs group was significantly higher than that of the OVA group. Treatment with ADSCs/HO-1 and ADSCs dramatically reduced RL and restored Cdyn compared with OVA group in response to MCH, respectively (**Figure 1**). But the effect of ADSCs with HO-1 was significantly better than that of ADSCs. These results suggested that HO-1 delivery via ADSCs significantly inhibited AHR.

Effect of HO-1 delivery via ADSCs on lung inflammation

The number of inflammatory nuclei around the airways, were significantly increased in OVA group mice, but significantly decreased after administration of ADSCs/HO-1 and ADSCs (**Figure 2A**). The scores for the peribronchial inflammation were significantly decreased in the ADSCs/HO-1 group and ADSCs group compared with the OVA group mice (**Figure 2B**). Numbers of total cells, as well as Eosinophils, Lymphocytes, Neutrophils, Macrophages in BALF in OVA group were significantly increased compared with NC group. Treatment with ADSCs/HO-1 and ADSCs significantly reduced numbers of total cells and all cell types in the BALF (**Figure 2C**), but the effects of ADSCs/HO-1 was significantly greater than those of ADSCs. These results suggested that the HO-1 delivery via ADSCs inhibited OVA-induced inflammation in the lung.

Effects of HO-1 delivery via ADSCs on cytokine levels in BALF

We evaluated the immunomodulatory effects of HO-1 delivery via ADSCs in the mouse asthma model. Mice in OVA group showed increased

levels of IL-4, IL-5, IL-13 and decreased levels of IFN- γ and IL-10 in BALF compared with NC group. IL-4, IL-5 and IL-13 levels were significantly reduced, However, IFN- γ and IL-10 levels in BALF was significantly increased in the ADSCs/HO-1 and ADSCs group compared with those in OVA group (**Figure 3**), but the effect of ADSCs/HO-1 was significantly greater than that of ADSCs. We concluded that HO-1 delivery via ADSCs decreased IL-4, IL-5, IL-13 levels and restored IFN- γ and IL-10 in BALF after OVA challenge.

Expression of HO-1 delivery via ADSCs in lung tissues

We examined expression of HO-1 protein in local tissues using immunohistochemistry analyses. It was revealed that expression of HO-1 is relatively low in NC group and higher in OVA group than that in NC group, treatment with ADSCs/HO-1 and ADSCs significantly increased expression of HO-1, however, the increase of expression in ADSCs/HO-1 group is more significant than that in ADSCs group (**Figure 4**).

Discussion

We first established a murine asthma model to evaluate the effects of HO-1 delivery via ADSCs on asthma allergic responses. In the current study, ADSCs were used to carry lung-specific gene transfer for asthma; we demonstrated that HO-1 gene transfer with ADSCs alleviated airway inflammation of OVA-sensitized mice. HO-1 expression level in OVA-sensitized mice was higher than that in NC group; HO-1 expression level in ADSCs/HO-1 group was increased after gene treatment compared with that in OVA-sensitized mice. In addition to the increment of IL-10 and IFN- γ in BALF, inflammatory cells infiltration, AHR and Th2 cytokines in BALF were significantly reduced in ADSCs with HO-1 gene-treated mice. These results indicated that HO-1 delivery via ADSCs might be a good candidate for the treatment of lung inflammation. It is also provide a strong support that HO-1 delivery via ADSCs could serve as a potential therapeutic method for the treatment of asthma.

HO-1 is an inducible isoform of the rate-limiting enzymatic step of heme catabolism and has important antioxidant and anti-inflammatory functions [19]. Several studies have shown that

the induction of HO-1 helps to ameliorate tissue injury and inflammation in a variety of experimental animal models and in humans [11, 20-22], suggesting that HO-1 induction serves to protect cells from injury or inflammation. Lee and collaborators demonstrated overexpression of HO-1 could decrease airway inflammation in the model of mouse [11], the protective effect was probably associated with IL-10 expression in CD4⁺CD25⁺Foxp3⁺ T regulatory cells and inhibiting the release of Th2 cytokines to suppress IgE and eosinophilia [23, 24]. The present study indicated that overexpression of HO-1 suppressed Th2 response and restored Th1 response in OVA-induced asthma model, these data suggested that the effect of HO-1 on Th1 and Th2 response might be useful for developing novel therapeutic approaches in treating asthma and other inflammatory diseases. Result of our study was consistent with Lee's [11]. Furthermore, in our study the expression and anti-inflammation of HO-1 would probably be long-lasting as the HO-1 gene delivery via lentivirus could encode into mice genome.

Asthma was characterized with a predominance of Th2 lymphocytes which generated plenty of IL-4, IL-5, and IL-13 in the airways [25]. These cytokines have been involved with eosinophilic airway inflammation and AHR in asthma patient and mouse asthma model [26]. IL-4 was the most important Th2 cytokine, it could induce isotype switching to IgE in B lymphocytes [27]. IL-4-deficient mice shown significant mild asthma feature and no obvious AHR [28]. Similarly, IL-5 played an important role in mediating eosinophil expansion, prolonged tissue survival and recruitment in response to allergen [29]. IL-4 and IL-5 were thus important regulators of airway inflammation in asthma. IL-13 level was also elevated in the airways and sputum of asthma patients [30]. IL-4 and IL-13 orchestrated inflammation associated with asthma, they were produced by lymphocytes as well as mast cells, eosinophils and macrophages [31]. Study reported that IL-13 was responsible for increased severity of asthma and elevated IgE level [32]. Furthermore, IL-13 could in turn increase susceptibility of chlamydial infection in lung that could theoretically develop a positive feedback loop to produce or make asthma worse [33]. We shown here that HO-1 delivery via ADSCs reduced the levels of IL-4, IL-5 and IL-13 in BALF, and the infiltration

of inflammatory cells in lung tissues. The decrease of IL-4, IL-5 and IL-13 was associated with the reduced infiltration of inflammatory cells in lung tissues.

IL-10 was an anti-inflammatory cytokine produced by Treg cells. IL-10 could inhibit the survival of eosinophils and attenuate the synthesis of Th2 cytokines, many proinflammatory cytokines and chemokines, such as IL-6, TNF- α , IgE and IL-8, particularly at the attack of asthma [34-36]. Antibody to IL-10 further exaggerated Th2-dominant airway hypersensitivity [37]. HO-1 might promote the number of CD4⁺CD25⁺ high Treg cells by increasing the secretion of IL-10 and TGF- β [36]. Some studies shown that transgenic expression of HO-1 cDNA and induction of HO-1 could significantly increase the production of IL-10 [38, 39]. The level of IL-10 in patients with asthma was lower than that in non-asthma individual [40]. But some studies also found a higher IL-10 level in individuals with asthma than in control individuals [8]. In this study, the level of IL-10 in OVA group was lower than that in NC group, it was consistent with Duvernelle's result [40]. We also found that the level of IL-10 in ADSCs/HO-1 was significantly higher than that in OVA group. This result strongly implied that overexpression of HO-1 delivery via ADSCs increased the produce of IL-10 to regulate inflammatory responses.

IFN- γ was produced by Th1 T lymphocytes [41], IFN- γ might also play a protective role in atopic asthma by functionally antagonizing IL-4-driven Ig isotype switching to IgE synthesis, and Th1 cytokines might also be considered protective in asthmatic airways [42]. The role of IFN- γ in asthma was controversial. Some studies reported that levels of IFN- γ in patients with asthma was lower than that in non-asthma individuals [43, 44]. But some other studies reported that IFN- γ level was increased in allergen challenge asthma model [45]. Our result indicated that IFN- γ level was significantly decreased in OVA group and consistent with Park's result [44], it was increased by overexpression of HO-1 delivery via ADSCs.

AHR, a key functional indicator for asthma, is involved with various cytokines such as IL-4 [46], IL-5 [47], IL-13 [48], IL-33 [49]. Circulating rather than airways IL-5 was responsible for the AHR [50]. Blockade of IL-13 or IL-5 by the administration of IL-13 receptor antagonist or

blocking antibodies to IL-5 inhibited airway AHR in animal models of asthma [51]. But blockade of IL-4 by the soluble IL-4 receptor only decreased the eosinophilia infiltration in lung without attenuating AHR in OVA-induced mice [52]. Overexpression of IL-10 have been shown to inhibit AHR induced by Th2 cells [53, 46]. Plasmids encoding with IFN- γ gene or transferred by an adenovirus vector could decrease and reverse established AHR induced by allergen, infiltration of airway eosinophilia and production of Th2 cytokine [54]. In present study, we found that overexpression of HO-1 decreased RL and restored Cydn, the effect on AHR was probably via reducing IL-4, IL-5, IL-13 and increasing IL-10, IFN- γ .

In conclusion, these results demonstrated that HO-1 delivery via ADSCs exerted anti-inflammatory effect and protected lung function in OVA induced allergic asthma model. Treatment with ADSCs encoding HO-1 in vivo markedly reduced the production of Th2-associated cytokines, the severity of AHR and infiltration of inflammatory cells in the lung. These data suggest that HO-1 delivery via ADSCs might mediate a negative feedback modulation of Th2-predominant allergic airway inflammation, and that the HO-1 delivery via ADSCs has therapeutic potential for the treatment of allergic asthma.

Acknowledgements

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Disclosure of conflict of interest

None.

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