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Original Article

The SDF-1/CXCR4/AKT signaling regulates bone marrow mesenchymal stem cell trafficking to promote airway remodeling in asthmatic rats

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Abstract: Stromal cell-derived factor-1 (SDF-1) is a potent chemokine for bone marrow mesenchymal stem cells (BMSC) by binding to its receptor, CXCR4. It was noted OVA-induced asthmatic rats manifested overexpression of SDF-1 and CXCR4, which were predominantly localized in the airways. We demonstrated experimental evidence indicating that the SDF-1/CXCR4 axis regulates BMSC trafficking to promote airway remodeling in asthmatic rats. As a result, blockade with AMD3100, a CXCR4 blocking antibody, significantly attenuated BMSC mediated airway remodeling in OVA-induced asthmatic rats. Moreover, administration of AMD3100 significantly repressed AKT activity, indicating that AKT is downstream of the SDF-1/CXCR4 axis. Collectively, our studies suggest that the SDF-1/CXCR4/AKT signaling could be a viable target for prevention and treatment of airway remodeling in asthmatic subjects.

Keywords: Bone marrow mesenchymal stem cells, SDF-1, CXCR4, airway remodeling, asthma

Introduction

Airway remodeling is a characteristic feature relevant to the pathoetiology of asthma, which includes epithelial damage, increased smooth muscle mass and the numbers of activated fibroblasts/myofibroblasts along with subepithelial fibrosis and vascular remodeling [1]. Despite past extensive studies, the mechanisms underlying airway remodeling are yet to be fully addressed. Bone marrow mesenchymal stem cells (BMSC) possess the properties for self-renewal and differentiation into various connective tissue lineages including adipose tissue, marrow stroma, cartilage and fibroblasts [2]. In ovalbumin (OVA)-induced asthmatic rats, the transition of smooth muscle cells into myofibroblasts is increased, likely to be pluripotent differentiation of mesenchymal stem cells induced differentiation of progenitor cells [3]. Similarly, allergen sensitization and challenge in mice are accompanied by the increase of BMSC residing in the lungs [4]. Collectively, these studies provided feasible evidence indicating a role for BMSC in airway remodeling during the course of asthma development.

CXCR4 and its ligand SDF-1, are constitutively expressed in a wide range of tissues, and are essential for normal development [5, 6]. They were recently found to be critical components relevant to inflammatory responses involved in allergic airway disease in animals [7]. Although the level of SDF-1 within the airways remained constant during the course of inflammatory response [7], CXCR4 inhibitors, however, reduced the hallmarks of allergic airway disease [8]. Given that SDF-1 is a chemotactic factor that may affects a number of physiological functions via binding to its receptor CXCR4 [4, 9-11], we thus hypothesized that SDF-1/CXCR4

signaling can exacerbate bronchia asthma and promote airway remodeling by recruiting BMSC into the inflammatory lung. To address this hypothesis, a neutralizing antibody was employed to block SDF-1/CXCR4 signaling in asthmatic rats. Our studies revealed that blockade of SDF-1/CXCR4 signaling significantly attenuated airway remodeling, which was associated with repressed recruitment of BMSC into the airways. Together, our data suggest that CXCR4 could be a viable therapeutic target for airway remodeling in clinical settings.

Materials and methods

Animals

Specific pathogen-free, female Sprague-Dawley rats (6-8 weeks old) were obtained from the experimental animal center of Guilin Medical University. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Guilin Medical University.

OVA-induced chronic asthma model

Chronic experimental asthma was induced in rats as preciously described [12]. Rats were sensitized with subcutaneous injection of 10 mg ovalbumin (OVA, Sigma, Shanghai, China) mixed with 200 mg alumin hydroxide solution on days 1 and 8. The rats were next exposed to the aerosolized OVA for 30 min per day for eight weeks starting from day 15. For controls, OVA was replaced with PBS during sensitization and challenge.

BMSC culture and administration

SD rat bone marrow-derived BMSC with ectopic green fluorescent protein (GFP) were purchased from Cyagen Biosciences (Guangzhou, China) and expanded according to manufacturer's instructions. BMSC were placed into 25 cm^2 culture flasks (Corning, NY, USA) and cultured with BMSC growth medium (Cyagen Biosciences, Guangzhou, China) at 37°C under 5% CO_2 and 90% humidity. The medium was changed every two days, and BMSC at the stage of fifth-eighth passages were used for experiments. Each experimental rat was administrated 1×10^6 BMSC in 1 ml PBS intravenously via an insulin syringe through tail vein once a week for 4 weeks.

Real time PCR

Total RNA was extracted from the lung with Trizol reagent. Real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed to analyze gene expression with the primers specific for SDF-1 (F: 5'-GCA TCA GTG ACG GTA AGC-3'; R: 5'-CTG AAG GGC ACA GTT TGG-3') and β-actin (F: 5'-AAG AGA GGC ATC CTC ACC CT-3'; R: 5'-TAC ATG GCT GGG GTG TTG AA-3') was used for normalization, and relative expression levels were determined using the $2^{-\Delta\Delta Ct}$ approach as reported [13].

Western blotting

Lung tissues were homogenized and lysed for 20 min on ice with RIPA lysis buffer containing protease inhibitor and PMSF, followed by centrifugation at 12,000 rpm at 4°C for 15 min. The supernatant was mixed with same volume of 4× protein buffer (Beyotime, Shanghai, China) and heated at 95°C for 5 min, then cooled down on ice for 5 min. The protein extract was separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred onto a polyvinylidene difluoride (PVDF) membrane. After blocking with 5% nonfat dry milk in TBS containing 0.1% Tween 20 for 2 h at room temperature, and the blots were incubated with indicated primary antibodies overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated secondary antibodies. The results were visualized with an enhanced chemiluminescence plus kit (Thermo, California, USA) as reported [14].

Immunostaining

Rats were sacrificed one week after last BMSC administration to collect trachea and lung tissues. The Lung was inflated, fixed with formaldehyde solution, and embedded with paraffin, then cut into 5-μm sections. The sections were de-paraffinated and hydrated with xylene and graded alcohol, and then pre-incubated in boiling sodium citrate buffer for antigen retrieval, followed by blocking with goat serum for 1 h at room temperature. The sections were next probed with a polyclonal antibodies against α-SMA and MCP-1 (Abcam, Cambridge, United States) overnight at 4°C, followed by staining with an Alexa Fluor 594-conjuated goat anti-mouse antibody, and were then mounted with

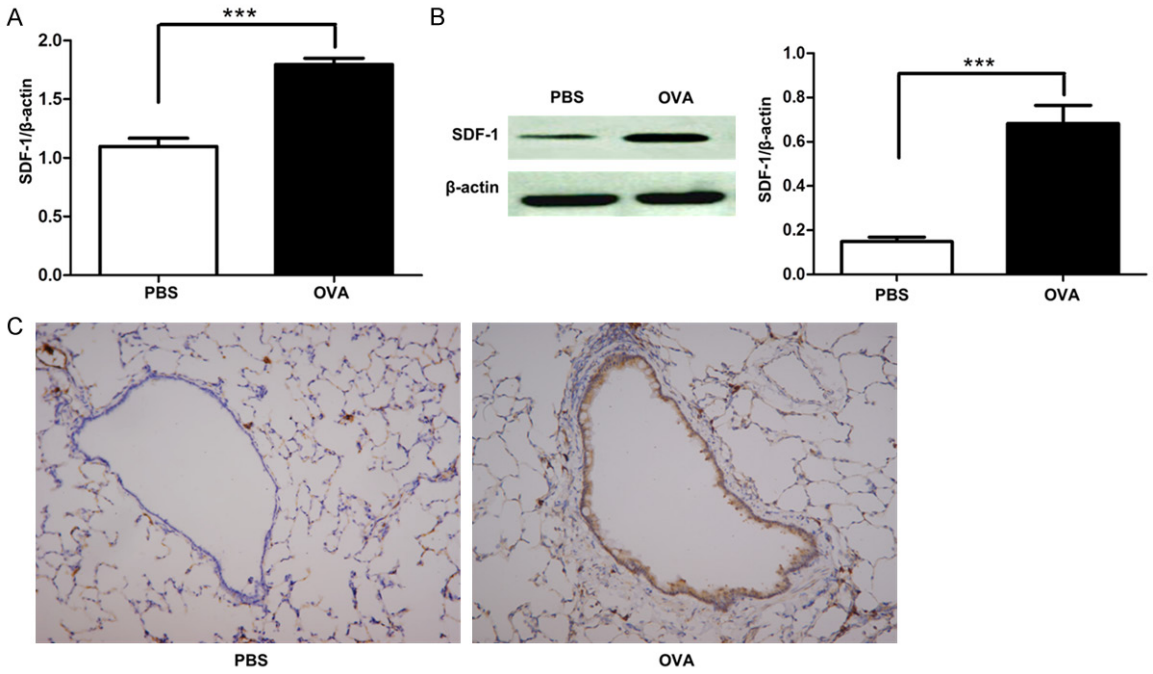

Figure 1. Lung tissues from asthmatic rats manifest enhanced SDF-1 expression. A. SDF-1 mRNA expression in OVA group and PBS group by real-time RT-PCR analysis. B. SDF-1 protein expression as determined by Western blotting. C. Immunohistochemical staining of SDF-1 in the airway walls. Images were taken under ×200 magnification (N = 6 per group). ***, $P < 0.001$.

mounting medium containing DAPI (Molecular Probes, California, USA) [15, 16].

Histological analysis

The above sections were subjected to hematoxylin and eosin (H&E) staining as reported [17, 18]. The sections were also subjected to periodic acid-Schiff (PAS) staining to examine the presence of mucin within the goblet cells, and Masson trichrome staining was conducted to assess the subepithelial layer as reported [19], and the immunohistochemical staining to examine SDF-1 distribution. The thickness of the subepithelial layer was defined by measuring the distance between the basement membrane and the lumial border of the tracheal cartilage and smooth muscle layer using Image Pro-plus system. Additionally, the mucus score was performed in a blinded fashion to determine the extent of mucus production using a 5-point grading system as follows: 0, no goblet cells; 1, < 25% goblet cells; 2, 25%-50% goblet cells; 3, 50%-75% goblet cells; 4, > 75% goblet cells. Mucus scoring was performed in at least three different fields for each lung section [20].

Statistical analysis

All experiments were conducted with 3 replications, and the data were expressed as mean ± standard error. Student's *t* test was employed to analyze the differences between two groups. Multiple comparisons were conducted by one-way or two-way ANOVA where appropriate followed by the Student-Newman-Keuls test [21]. In any case, $P < 0.01$ was considered with statistical significance.

Result

Lung tissues from asthmatic rats manifest enhanced SDF-1 expression

We first examined SDF-1 expression levels in asthmatic rats. It was interestingly noted that allergen sensitization and challenge resulted in a significant increase for SDF-1 mRNA levels as determined by RT-PCR analysis (**Figure 1A**). To confirm this observation, we conducted Western blot analysis of lung lysates, and similar results were obtained (**Figure 1B**). Next, lung sections were subjected to immunohistological analysis of SDF-1 positive cells in the airway of rats treated with PBS or OVA. Remarkably,

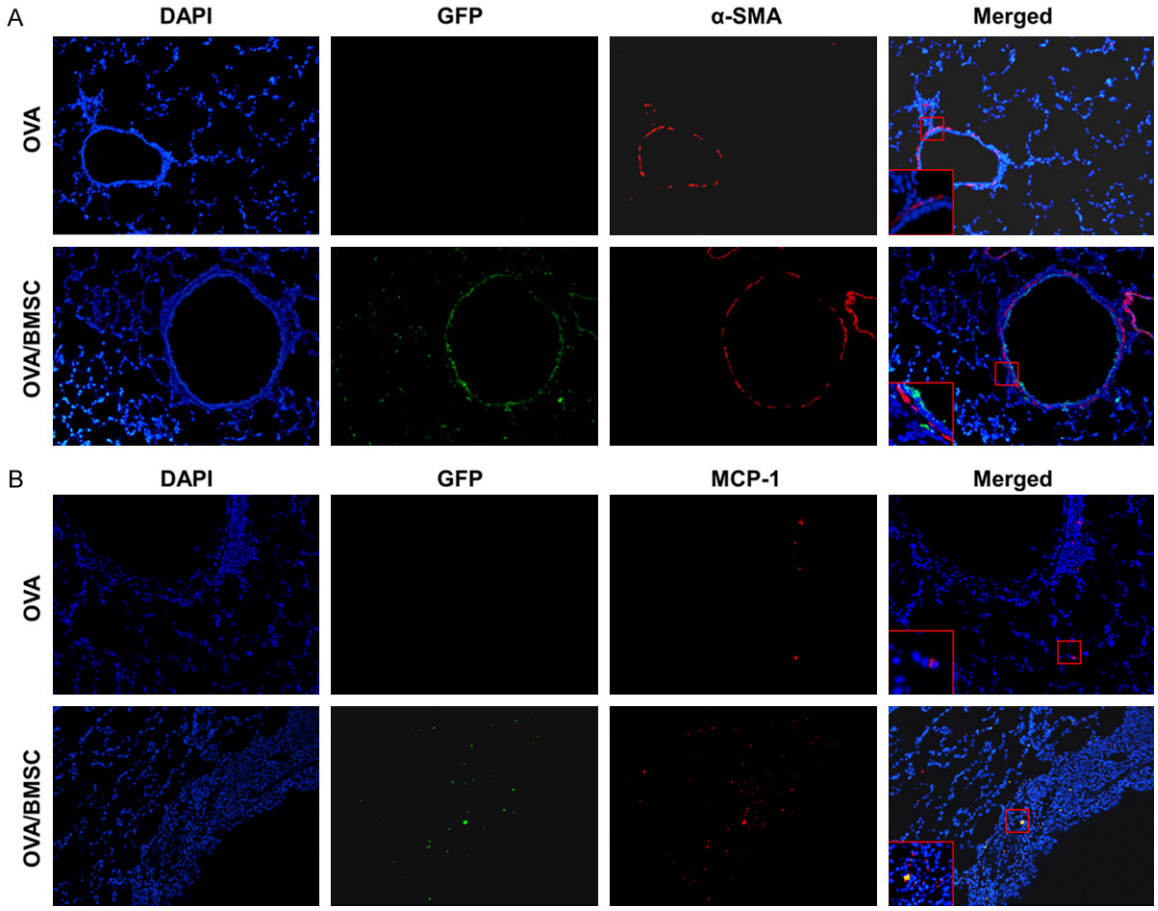

Figure 2. BMSC exacerbate airway remodeling in asthmatic rats. A. Results for GFP positive BMSC co-stained α-SMA (red) in the lung sections of OVA challenged rats. Nucleoli were stained with DAPI (blue). B. Lung sections were co-stained with MCP-1 (red) of OVA challenged rats injected with GFP-BMSC. DAPI (blue) was employed to stain nucleoli. Images were taken under ×200 magnification (N = 6 per group).

SDF-1 was almost undetectable in the airways originated from control rats, while high-levels of SDF-1 were detected in the airways of OVA-treated rats (**Figure 1C**). Particularly, SDF-1 was predominantly expressed in the bronchial epithelium ($P < 0.01$). Together, these data demonstrate that asthma manifests altered SDF-1 expression in the airways.

BMSC exacerbate airway remodeling in asthmatic rats

Given that SDF-1 signaling is critical for mesenchymal cell chemotaxis and organ-specific homing in injured tissues [22], and allergen sensitization and challenge is accompanied by the increase of BMSC [23-26], the above data prompted us to check whether SDF-1 signaling is involved in the trafficking of BMSC to lungs during airway remodeling, To address this question, we first injected GFP-BMSC via tail veins into asthmatic rats, followed by microscopic analysis of GFP-BMSC in the lung sections. Indeed, GFP-BMSCs were characterized in the airway as manifested by the GFP expression and co-staining of α-SMA (**Figure 2A**).

The next important question is whether the aggregation of BMSC promotes airway remodeling. To this end, we first conducted MCP-1 immunostaining, and noted that GFP-positive BMSC expressed high levels of MCP-1 (**Figure 2B**). To further address this question, we compared inflammatory infiltration, mucin production within the goblet cells, and subepithelial thickness between asthmatic and GFP-BMSC injected asthmatic rats. Remarkably, the inflammatory severity (**Figure 3A**), mucin production (**Figure 3B**), and subepithelial thickness (**Figure 3C**) were significantly increased in BMSC injected asthmatic rats as compared with that of asthmatic and normal rats, suggesting that the

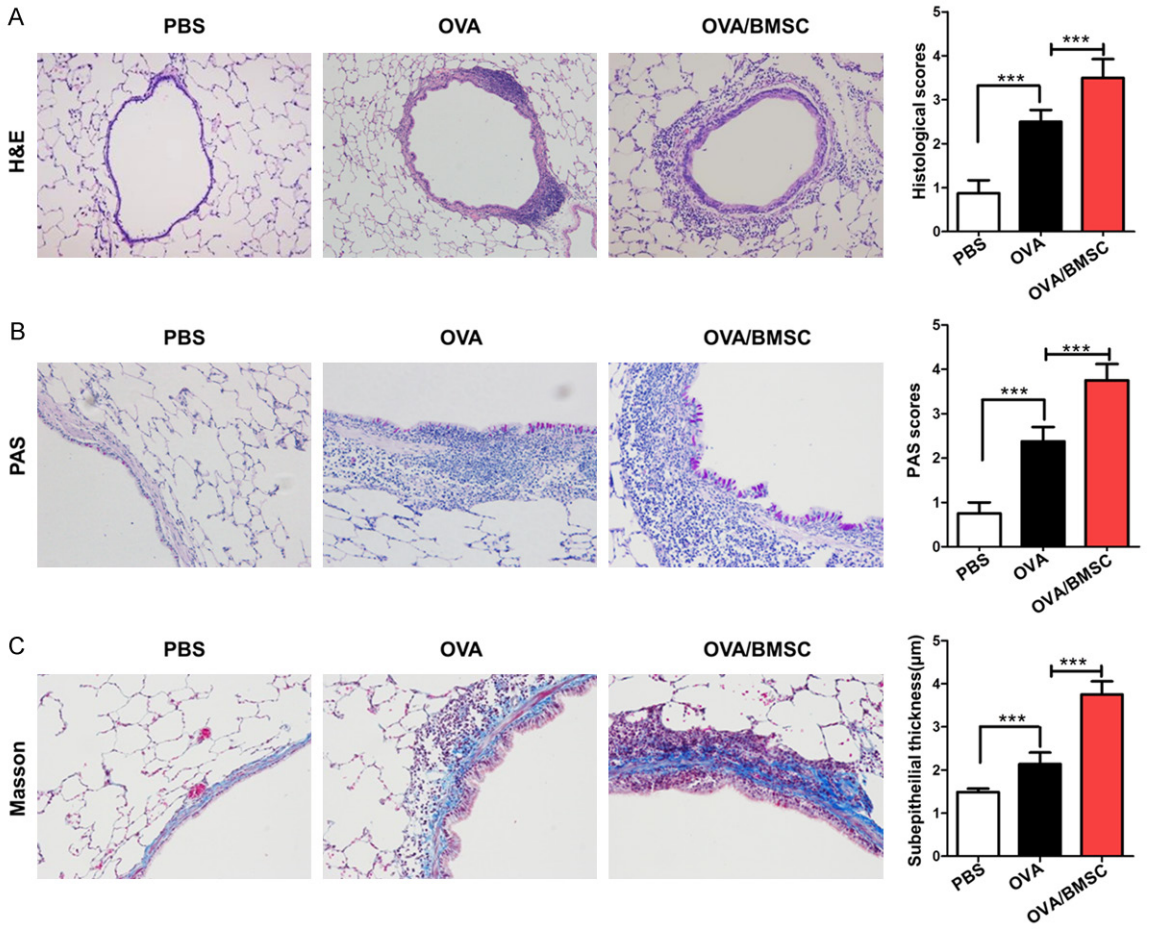

Figure 3. BMSC exacerbate airway remodeling in asthmatic rats. A. H&E-stained lungs show histological scores. B. PAS staining show mucin production within the goblet cells. C. Masson trichrome staining for assessing subepithelial thickness. Images were taken under ×200 magnification (N = 6 per group). *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

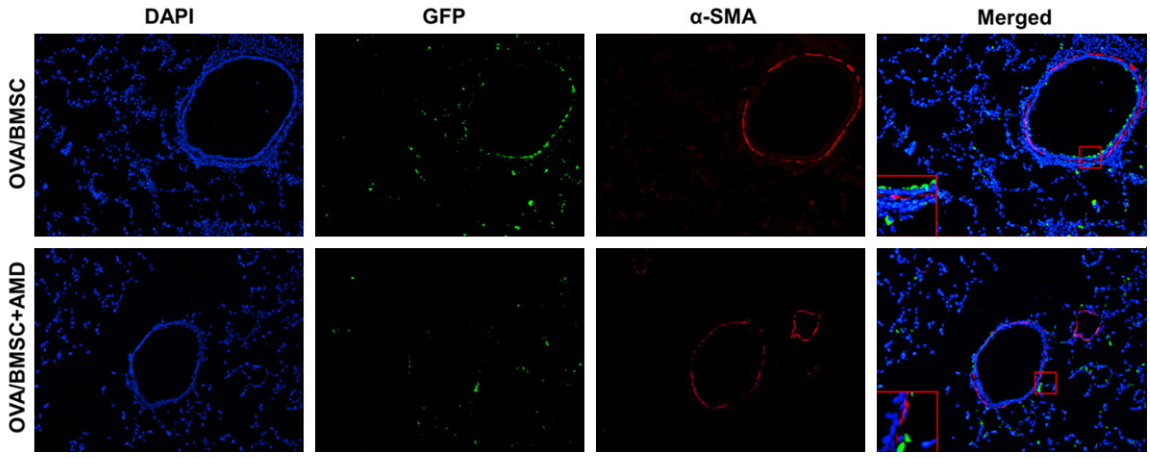

Figure 4. BMSC enhance SDF-1/CXCR4/AKT signaling to promote airway remodeling. Administration of CXCR4 blocking antibody, AMD3100, significantly attenuated BMSC trafficking into the airways. GFP indicates BMSC, α-SMA was stained in red, and nucleoli were stained by DAPI in blue. Images were taken under ×200 magnification (N = 6 per group).

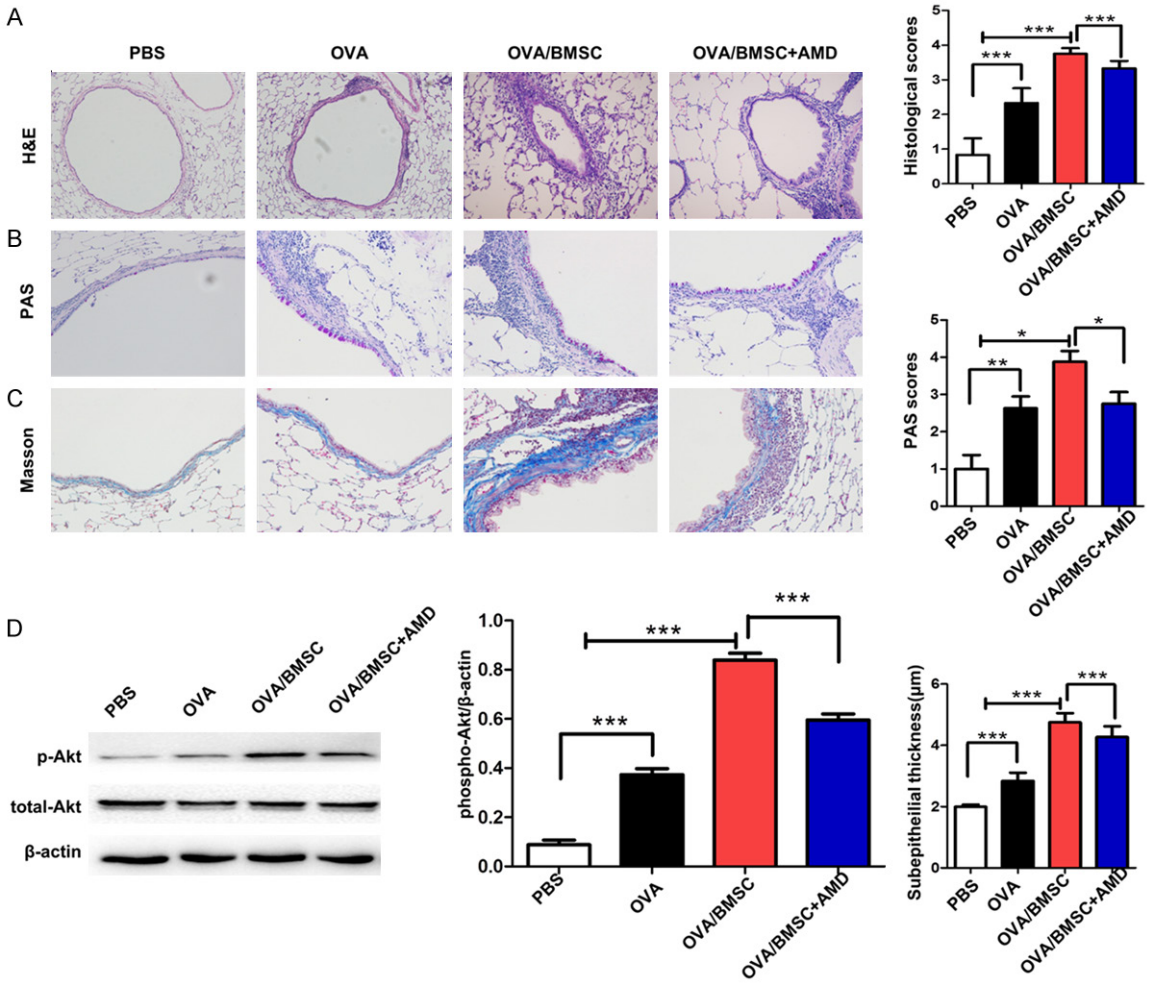

Figure 5. BMSC enhance SDF-1/CXCR4/AKT signaling to promote airway remodeling. A. Results for H&E staining of lung sections. B. PAS staining results for assessing mucin production within the goblet cells. C. Masson trichrome staining results for analysis of subepitheilial thickness. Images were taken under x200 amplification (N = 6 per group). D. Western blot analysis of AKT activity. Original magnification ×200 (N = 6 per group). *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

injected BMSC aggregated into the airway, through which, they exacerbate airway remodeling.

BMSC enhance SDF-1/CXCR4/AKT signaling to promote airway remodeling

To dissect the mechanisms by which BMSC promote airway remodeling, we administered AMD3100, a non-toxic blocking antibody for CXCR4 which is the receptor for SDF1, before injection of BMSC into the asthmatic rats. Immunostaining of lung sections revealed that blockade of CXCR4 by AMD3100 significantly attenuated BMSC trafficking into the airway (indicated by α-SMA staining) as manifested by the reduced number of GFP positive BMSC (**Figure 4**). In line with this observation, administration of AMD3100 significantly repressed BMSC-mediated inflammatory infiltration (**Figure 5A**), mucin production (**Figure 5B**), and subepithelial thickness (**Figure 5C**) after OVA induction. It has been noted that allergen sensitization and challenge are associated with increased AKT activity [27], We thus further examined phosphorylated AKT (p-AKT) levels. Indeed, injection of BMSC significantly increased AKT activity as manifested by higher levels of p-AKT as compared with that of asthmatic rats without BMSC injection, and more importantly, blockade of the SDF-1/CXCR4 axis by AMD3100 significantly repressed the effect of BMSC on AKT activity (**Figure 5D**), indicating that AKT is downstream of SDF-1/CXCR4 signaling, by which it regulates BMSC trafficking

implicated in airway remodeling in asthmatic rats.

Discussion

In the present study, we assessed the functional significance of SDF-1/CXCR4 signaling in BMSC trafficking into the airways of asthmatic rats. Particularly, we demonstrated that BMSC can be recruited into the lungs after allergen sensitization and challenge, in which SDF-1/CXCR4 signaling plays an essential role. It was noted that SDF-1 is constitutively expressed in allergic lung relevant to airway remodeling. Furthermore, our studies revealed that airway remodeling was associated with the accumulation of injected GFP-BMSC in the airways of asthmatic rats. Therefore, blockade of SDF-1/CXCR4 signaling by a CXCR4 neutralizing antibody significantly attenuated BMSC mediated airway remodeling, suggesting that the SDF-1/CXCR4 axis could be a viable therapeutic target for preventing airway remodeling in asthmatic subjects.

SDF-1 has previously been noted to be increased in bronchial mucosa in asthmatic subjects [28]. Particularly, SDF-1 and CXCR4 are preferentially expressed by the bronchial epithelium [29, 30], which may be associated with increased subepithelial layer [31] and luminal myofibroblasts observed in asthmatic subjects [32]. Indeed, our studies in asthmatic rats revealed that SDF-1 was overexpressed in the allergic lungs, which then promotes airway remodeling.

BMSC are multipotent cells capable of self-renewal and differentiation into a selective range of mesenchymal cell types dependent on the microenvironment [24]. Previous studies demonstrated feasible evidence suggesting the involvement of BMSC in asthma pathoetiology [24, 33]. We now provided experimental evidence that BMSC were recruited into the lungs, which then promote airway remodeling as manifested by the increased perivascular and peribronchial mononuclear cell infiltration, mast cell and globet cell hyperplasia and hypertrophy. BMSC can secrete a wide range of cytokines and growth factors to modulate injury microenvironment and improve repair/remodeling [33, 34]. During the course of chronic asthma, inflammatory stimulation renders BMSC preferentially differentiating into myofibroblast to promote the development of airway remodeling [35]. A large number of inflammatory cytokines can stimulate BMSC differentiating into epithelial cells and fibroblasts to participate injury repair in the lung. Mesenchymal stem cells also secrete a number of vascular endothelial growth factors such as angiopoietin-2, fibroblast growth factor, and stem cell growth factors involved in generating vascular smooth muscle, thereby aggravating asthma airway remodeling [36]. Indeed, our studies revealed that BMSC accumulated in the airway express high levels of MCP-1, and asthmatic rats injected with BMSC were manifested by the increased inflammatory infiltration, mucin production and subepithelial thickness.

Stromal cell-derived factor-1 (SDF-1) and its receptor, CXCR4, are crucial for homing and migration of multiple stem cell types [9]. To investigate the role of SDF-1/CXCR4 signaling in the recruitment of BMSC into allergic lungs relevant to airway remodeling, a CXCR4 neutralizing antibody, AMD3100, was employed to block the SDF-1/CXCR4 signaling in BMSC. Remarkably, blockade of SDF-1/CXCR4 signaling significantly attenuated the trafficking of GFP-BMSC into allergic airways. However, a small proportion of BMSC was still recruited into the allergic airway in the presence of AMD3100, indicating that additional migration-stimulating factors other than SDF-1/CXCR4 signaling are also involved in the trafficking of BMSC. Given the role of AKT signaling played in pathoetiology of asthma [37], we further examined the impact of AMD3100 on AKT activity. Remarkably, pre-treatment of rats with AMD-3100 significantly attenuated the impact of BMSC on airway remodeling in asthmatic rats, indicating that AKT is downstream of SDF-1/CXCR4 axis. Together, our data support that SDF-1/CXCR4/AKT signaling plays a critical role in BMSC recruitment during the course of airway remodeling in asthmatic setting.

In conclusion, we demonstrated that asthmatic rats manifest overexpression of SDF-1, which then promotes BMSC recruitment to facilitate airway remodeling. As a result, blockade of the SDF-1/CXCR4 axis by AMD3100 significantly attenuated BMSC recruitment along with alleviated airway remodeling. Mechanistic studies revealed AKT is downstream of SDF-1/CXCR4 axis, by which it manifests an essential role in BMSC mediated airway remodeling in asthmat-

ic setting. Collectively, our data suggest that the SDF-1/CXCR4 axis could be a viable target for prevention and treatment of airway remodeling in asthmatic subjects.

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Disclosure of conflict of interest

None.

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