Smooth muscle progenitor cells involved in the development of airway remodeling in a murine model of asthma

Yongfu Wu,* Hui Fu,* Huiling Yang, Hongqiang Wang, Hanbin Zhang and Dongyun Qin

Summary

Background: **The mechanisms regulating airway remodeling changes remain poorly understood. Recently, a smooth muscle progenitor cell was identified in the peripheral circulatory system that plays an important role in the reconstruction of injured blood vessels. However, to the best of our knowledge, there is no report in the medical literature regarding the role of smooth muscle progenitor cells (SPCs) in asthma.**

Objective: **The aim of this study was to investigate the relationship between SPCs and the development of airway remodelling in a murine model of asthma.**

Methods: **Chronic asthma with airway remodeling was generated by sensitizing and stimulating BALB/c mice with atomized ovalbumin (OVA). Bronchoalveolar lavage fluid (BALF) was collected for eosinophils (EOS) counting and histological analysis. The Ficoll method was used to isolate mononuclear cells from peripheral blood. Smooth muscle myosin heavy chain (SM-MHC) and highly glycosylated type I transmembrane protein ($CD34^+$) were selected as two markers to detect the expression of SPCs by Flow Cytometry.**

Results: **Long-term inhalation of OVA produced thickening of the epithelial and smooth muscle layer, goblet cell hyperplasia, collagen deposition around smooth muscle, luminal exudates and inflammatory cell infiltration. The number of SPCs in the asthma group was significantly higher than in the control group.**

Conclusion: **Long-term inhalation of OVA results in airway remodeling and the smooth progenitor muscle cell are involved in the development of airway remodeling.** ***(Asian Pac J Allergy Immunol 2014;32:203-10)***

Keywords: *Asthma, airway remodeling, SM-MHC, CD34, smooth muscle progenitor cell*

Introduction

The term airway remodeling refers to structural changes which include inflammatory cell infiltration, subepithelial fibrosis, epithelial hyperplasia and metaplasia, muscle cell hyperplasia and angiogenesis.[1] These changes result in thickening of the walls of the airway causing fixed airflow obstruction, increased airway hyper-responsiveness (AHR) and irreversible loss of lung function.[2-4] The mechanisms regulating these changes are still unclear. Research on the mechanisms of airway remodelling in asthma mainly focuses on repeated episodes of allergic inflammation; defective epithelial repair and mechanical stress.[5] Currently, asthma therapies are mainly designed to reduceg inflammation, such as inhaled corticosteroids, antileukotrienes and theophylline,[6-7] but cause many side-effects and cannot completely eliminate AHR or resolve airway obstruction. For this reason, an understanding of the mechanisms of airway remodeling is required.

Recently, a new type of cell, the smooth muscle progenitor cell (SPCs), has been identified from peripheral blood.[8] It is currently thought that SPCs are precursor cells that can directly differentiate into smooth muscle cells *in vivo* and *in vitro*.[9] Identification of SPCs is an important scientific advance in the area of vascular remodeling.[10] However, the relationship between SPCs and the development of airway remodeling in asthma is still unknown. It may be an important discovery, because previous studies on asthma observed unique migration, hypertrophy and proliferation of smooth muscle cell in the airway, but the underlying

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mechanisms are not clear. Understanding the phenotype of SPCs in the airway may have implications for development of novel therapies to treat airway remodeling. Although more and more studies have revealed the features of SPCs, researche on SPCs is still in its infancy. Based on this, we hypothesized that SPCs in the peripheral blood may be increased and involved in the development of airway remodeling. To test this hypothesis, we tried to elucidate whether the number of SPCs among peripheral mononuclear cells changed in mice with asthmatic airway remodeling.

Methods

Experimental animals

Specific pathogen free (SPF) BALB/c mice (Age: 6-8 week old; Gender: female; Weight: 20±2g) were provided by the Experimental Animal Center of Guangdong Medical College. The mice were individually caged at a temperature of 24-28degrees C and a humidity of 50%-60%. The mattresses in the cages were changed every other day. Both water and food (standard feeds provided by the Animal Center) were freely accessible by the mice.

Sensitization and inhalational exposure

Modified protocols for rat sensitization and challenge were used as reported previously.[11] Forty BALB/c mice were randomly divided into a control group and an experimental group. In the control group, the mice were sensitized by intraperitoneal and multiple hypodermic injections of 2.5 ml normal saline containing 2.5 mg Aluminum hydroxide gel on days 0, 7 and 14. From day 21, the mice inhaled atomized normal saline for 30 min every day, three days a week for 8 weeks. In the asthmatic group, the mice were sensitized by intraperitoneal and multiple hypodermic injections of 0.5 ml normal saline containing 10 μg OVA and 2.5 mg Aluminum hydroxide gel on days 0, 7 and 14. From day 21, the mice inhaled atomized normal saline containing 1% OVA for 30 min every day, three days a week for 8 weeks. During the stimulation, the mice were placed in a box and the content of atomization was stabilized at 2ml/min by manual adjustment.

Sample collection and treatment

Forty-eight hours after the last stimulation, the mice were anaesthetized and 1ml blood was collected from the eyeball. Peripheral mononuclear cells were isolated by Ficoll density gradient centrifugation and stored in a refrigerator at 4°C. Flow cytometry was conducted within 6 hours after the samples were collected. The mice were euthanized and the chest was opened followed by ligation of left porta pulmonis. The left lung was cut off and fixed with 10% formaldehyde for 24 h. After fixation, the sample was embedded in paraffin. Lavage was performed on the remaining right lung.

Eosinophil (EOS) counting

After ligation of the left porta pulmonis, a "T"-shaped incision was made in the lower part of trachea. 22G intravenous catheter tube was used for endotracheal intubation. When the needle was inserted, the stylet was pulled out. The needle was further pushed forward for 0.5 cm and fixed with thread. PBS (1 ml) was injected slowly through the plastic tube with a syringe. Once the right lung was expanded, the syringe was maintained for 1 min and then slowly pulled back. This process was repeated 3 times and a total of 3 ml PBS was injected. The recovery rate of the lavage fluid was > 85%. The lavage fluid was centrifuged for 5 min at 2000 g and resuspended in 2 ml PBS for EOS counting within 3 h.

Isolation of peripheral mononuclear cells: Ficoll density gradient centrifugation

Anticoagulated blood (1 ml) was mixed with Hank's solution at a ratio of 1:1. The mixture was then carefully added to the surface of a cell separation solution (2 ml) (340 g/l diatrizoate was added to the Ficoll solution to obtain a separation solution with a density of 1.088). After centrifugation for 15 min at 2000 g, the cells on the top of the interface were collected and added to the tube containing 2 ml of Hank's solution. After complete mixing, the samples were centrifuged for 20 min at 2000 g and the supernatant was collected. The pellet was washed twice and resuspended in 0.6 ml Hank's solution for flow cytometry.

Flow cytometry

For detection of $CD34^+$ cells, two tubes labeled with "test" and "control" were filled with 0.1 ml cell suspensions. In the test tube, 10 μl of anti-mouse CD34 antibodies from rats (SANTA CRUZ, CA) was added. The control tube did not have antibodies added. The tubes were incubated at room temperature in a dark room for 25 min and then 0.5 ml PBS was added to both the test and control tubes. After centrifugation for 5 min, the supernatant was discarded. The pellet was added to 0.5 ml BSA (1.5%) to block non-specific antibody binding. Subsequently, FITC-conjugated goat anti-rat secondary antibody was added and the tube was

incubated at room temperature in a dark room for 20 minutes. 0.25 ml PBS was added to resuspend the cells by votexing, followed by flow cytometry analysis. For detection of SM-MHC$^+$ cells, two tubes labeled with "test" and "control" was filled with 0.1 ml of cell suspensions. Fixing reagent (50 μl) was added to both tubes to resuspend the cells by votexing. After incubation at room temperature in a dark room for 20 min, 0.5 ml buffer was added and the tube was centrifuged for 5 min at 1500 g. The supernatant was discarded and the pellet was added to 50 μl buffer to resuspend the cells. Rat antibody against mouse MHC-PE (SANTA CRUZ, CA) (5 μl) was added to the test tube. The antibody was not added to the control tube. After incubation at room temperature in a dark room for 20 min, 0.25 ml PBS was added to resuspend the cells by votexing. Flow cytometry analysis was subsequently performed. For detection of CD34$^+$/SM-MHC$^+$ cells, two tubes labeled with "test" and "control" were filled with 0.1 ml of cell suspensions. In the test tube, 10 μl of rat antibodies against mouse CD34 was added. The antibody was not added to the control tube. After incubation at room temperature in a dark room for 20 min, 0.5 ml PBS was added and the tube was centrifuged for 5 min at 1500 g. The supernatant was discarded and the pellet was added to 0.5 ml BSA (1.5%) to block non-specific antibody binding. FITC-conjugated goat anti-rat secondary antibody was added and the tube was incubated at room temperature in a dark room for 20 min. Fixing reagents (50 μl) were added to resuspend the cells by votexing. Penetrating buffer (0.5 ml) was added and the tube was centrifuged for 5 min at 1500 g. The supernatant was discarded and the pellet was added to 50 μl penetrating buffer to resuspend the cells. Rat antibody (10 μl) against mouse CD34 was added to the test tube, but not to the control tube. After incubation at room temperature in a dark room for 20 min, 0.5 ml penetrating buffer was added and the tube was centrifuged for 5 min at 1500 g. The supernatant was discarded and the pellet was added to 0.25 ml PBS to resuspend the cells. Flow cytometry was performed subsequently. For each tube, 5000 nucleated cells were detected. The gate was set by using each cell population in the FSC/SSC scatter graph of the control tube. This scale was used to determine the negative area of the histogram in each control and the percentage of the cell population in the histogram of each test tube was analyzed.

Morphometrical analysis

Paraffin-embedded samples were placed in the section machine and the position of the samples was adjusted to ensure that the transection was made from the left porta pulmonis (opening site) vertically against the ordinate axis of the lung. The total μm (∑μm) was recorded when the lung tissues were sectioned. At least 3 sections with a thickness of 5 μm/section were cut from a ∑μm of 1000 μm to 1500 μm (Figure 1, left main bronchus can be

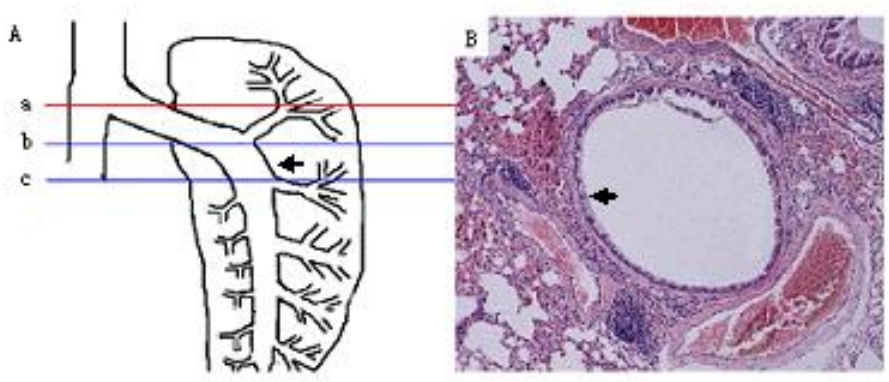

Figure 1. Schematic representation of sample collection and parameter measurment
(A) The "a" line indicates the site where the porta pulmonis was cut open. The region between line "b" and line "c" were the sites where lung sections were collected. This region was derived by extension of porta pulmonis. The distance between line "a" and line "b" was approximately 1000 μm. The distance between line "a" line and line "c" line approximately 1500 μm. At least 3 paraffin sections with a thickness of 5 μm/section were collected line "b" line and line "c". The arrow in the figure indicates left main bronchus. (B) One large bronchus (arrow) and three associated large vessels (one pulmonary artery and two pulmonary veins) can be observed in the transection. This bronchus was selected for the morphometrical analysis.

observed in each section). HE, AB-PAS and Masson staining was performed for each section. AB-PAS-positive goblet cells were stained blue. Masson staining of collagen fibers was blue. Functional modules (Irregular AOI and Segmentation) in the software Image-Pro Plus 6.0 were used to measure the thickness of the left main bronchial epithelial layer (Re), the thickness of smooth muscle layer (Rm), the number of goblet cells positively stained by AB-PAS, the area of the goblet cells (A_G, excluding the positively stained mucous area) and the percentage of goblet cell area among the area of epithelial layer ($\%A_G$). The thickness of the epithelial and smooth muscle layers was determined by calculating the average radius, based on the area of the epithelial and smooth muscle layers. The region that has an exradius along the trachea with collagen deposition was selected. The area with collagen deposition and the total area within the selected region were determined. The percentage of the collagen deposition area within the total area in the selected region was calculated ($\% A_{CO}$).

Statistical analysis

The results from each experiment were expressed as mean ± S.E.M. A software package (SAS9.0) was used for statistical analysis. One-way ANOVA was used for comparisons between different groups. Differences were considered to be statistically significant when $P < 0.05$.

Results

General observations of the mice in the asthmatic and control groups

After inhalation of OVA, the mice in the asthmatic group displayed dysphoria and increased activities during the initial 10 min. In the following 10 min, the mice lay quietly with erect hair and shortness of breath. Severely affected mice had stretching of the neck, shrinking of the chest, shrinking of the abdomen, hyperpnea and incontinence. These symptoms did not appear in the mice in the control group.

EOS counting and Morphometrical analysis

The EOS in the asthmatic mice was $33.60\pm9.29\times10^4$/ml, which was significantly higher than that in the control mice ($1.75\pm0.46\times10^4$/ml) ($P < 0.05$). Morphometrical analysis showed that the thickness of the airway epithelial layer was 27.92±6.72 μm, the thickness of the smooth muscle layer was 16.52±3.24 μm, the number of goblet cell was 130.90±16.55, goblet cell area accounted for 18.31±6.88% of the total epithelial layer area, and the area with bronchial deposition of collagen accounted for 3.73±0.59% in the asthmatic mice. These histological parameters in the control mice were 16.01±2.16μm, 11.30±1.21μm, 7.80±2.30, 0.50±0.15% and 1.30±0.48%, which were significantly lower than those in the asthmatic mice. ($P < 0.001$) (Figure 2).

Pathological changes in bronchus and lung tissues

Bronchopulmonary sections were examined under the light microscope for the mice in both groups. For the mice in the asthmatic group, the bronchus showed significant changes. The airway epithelium was swollen and the smooth muscle layer was thickened. We also observed goblet cell hyperplasia, submucosal gland hypertrophy, exudation into the lumen, infiltration of inflammatory cells, mucus plug formation and collagen deposition around smooth muscle. These changes were not observed in the control mice (Figure 2, Figure 3).

Flow cytometry analysis

The percentage of $CD34^+$ or MHC^+ cells in the peripheral mononuclear cells in the group of asthmatic mice was 1.20±0.16% and 11.92±1.80%, respectively, which was significantly higher than that in the control mice (0.29±0.11% and 1.66±0.21%, respectively) ($P<0.05$). The percentage of both CD34 and MHC positive cells in asthmatic mice was 0.99±0.09%, which was also significantly higher than that in the control mice (0.21±0.10%) ($P < 0.05$). (Figurer 4, and Figure 5)

Discussion

In this study, all the BLAB/c mice sensitized by OVA developed varying degrees of asthma following stimulation by inhalation with the same allergen. Pathological examination revealed that the lung tissue of mice in the asthma model group had swelling of the airway epithelium, thickening of smooth muscle and large numbers of inflammatory cells in the airway wall and its surrounding tissues. The analysis of the results shows that the morphometrical parameters of the asthmatic group were significantly higher than those for the control group. These were consistent with the reported pathological changes in the asthmatic airway remodeling.[12-13] At the same time, the EOS count in BALF was significantly increased in the asthmatic mice. Therefore, this study successfully established an asthmatic airway remodeling model in BALB/c mouse. A number of studies have involved collection of different parts of the lung for

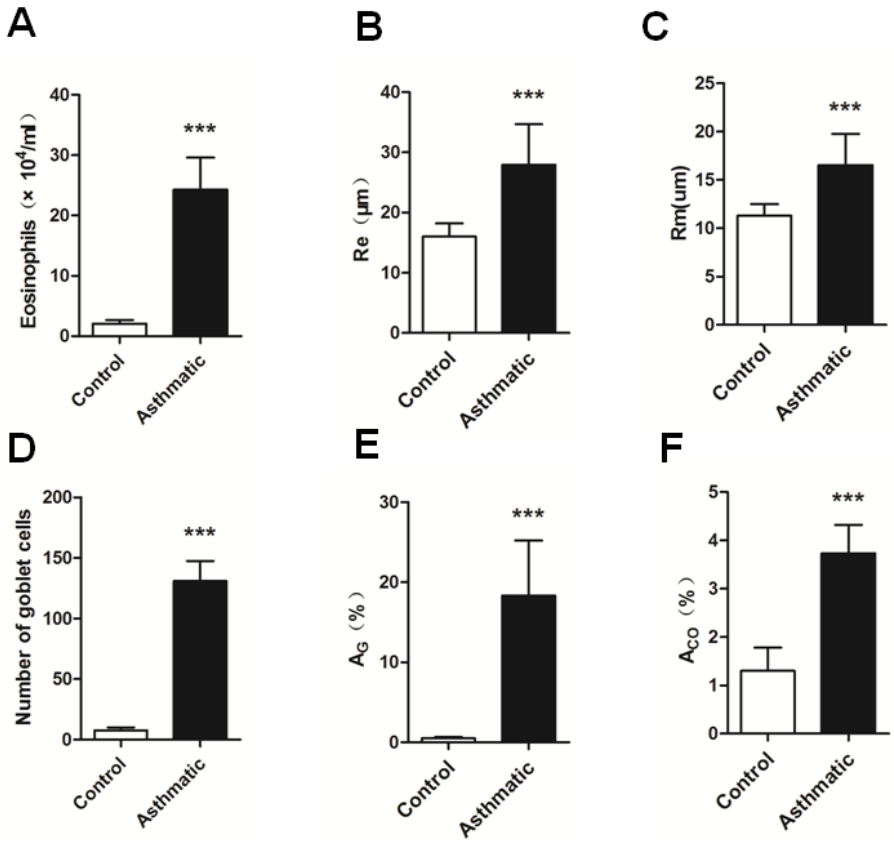

Figure 2. Eosinophil (EOS) counting (A) and characteristic histology for the bronchus and lung tissue in the asthmatic and control groups. (B) The thickness of the smooth muscle layer; (C) the thickness of the epithelial layer; (D) the number of goblet cells (E) the percentage of the goblet cell area within the total epithelium area; (F) the percentage of areas with collagen deposition surrounding the bronchus. The left main bronchus was used for all the parameter measurement. HE staining was used for measuring Rm and Re. AB-PAS section was used to measure $\%A_G$. Mason stained section was used for measuring $\%A_{co}$. ***P<0.001 compared to the control group. Date presented as mean ± S.E.M of 20 animals.

histological analysis.[14-16] These studies did not guarantee that the number and type of bronchus in each section were the same. The right lung of the mouse is divided into four lobes, while the left lung only has one lobe. The novelty of this study lies in the effective positioning of the left lung (Figure 1) by utilizing the external marker of the porta pulmonis followed by preparation of sections in a fixed range of tissues. The region that was selected for morphometrical analysis only contained one left bronchus. Thus, the procedures are simpler, repeatable and comparable.

SPCs are the most important discovery in the field of vascular remodeling in recent years. The current view is that smooth muscle cells, or at least a substantial part of the smooth muscle cells in the damaged tissue of atherosclerosis are most likely derived from their peripheral precursors, SPCs.[17-18] The role of SPCs in the asthmatic airway remodeling is not clear. Understanding the phenotype of SPCs may have implications for the development of novel therapies to treat airway remodeling. The developmental origin of SMPC is still not clearly determined. It is possible that neural crest and mesoderm-derived structures are two sources of SMPC.[19-22] Recently, more and more studies show that SPCs may originate from pluripotential stem cell in the bone marrow.[2, 23-25]At present, the specific cell surface antigens and the intracellular markers for the identification of SPCs have not been identified. Several contractile and other specialized smooth muscle proteins, such as smooth muscle a-actin (aSMA), SM22a, smooth musclemyosin heavy chain (SM-MHC), calponin, caldesmon and smoothelin have been used to identify SPCs.[26-28]Among these markers, SM-MHC seems to be the most specific for the SMC type.[2] Progenitor markers stem cell antigen CD34, foetal

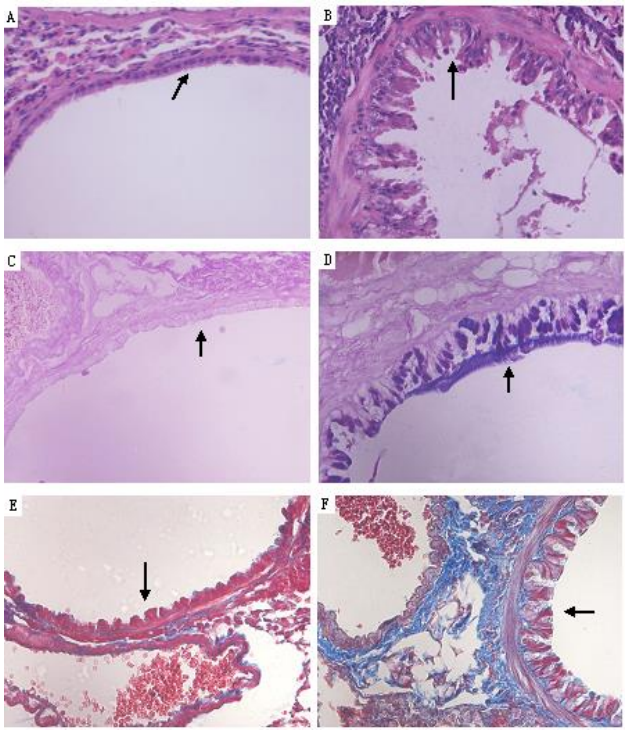

Figure 3. Pathological changes of the bronchus and lung tissues in the asthmatic and control mice (×400). The pathology of the control mice is shown in panels A, C and E. The pathology of the asthmatic mice is shown in panels B, D and F. HE staining is shown in panels A and B, AB-PAS staining is shown in panels C and D. Masson staining is shown in panels E and F. "Arrows" in the figure indicated the left main bronchus. Compared to the control mice, the bronchii of asthmatic mice showed airway epithelial swelling, smooth muscle layer thickening, goblet cell hyperplasia, submucosal gland hypertrophy, luminal exudate and inflammatory cell infiltration. In addition, the formation of mucus plugs and collagen deposition surrounding the smooth muscle can be observed.

liver kinase 1(flk1) and CD133 have also been used to identify SPCs.[29-30] It is known that SPCs have the characteristics of both smooth muscle and stem cells. However, different scholars have different views on the markers expressed by SPCs. In this study, we selected CD34 and SM-MHC as the two markers to detect SPCs in asthmatic and control mice by flow cytometry.

Flow cytometry analysis showed that $CD34^+$, $SM\text{-}MHC^+$ or $CD34^+/SM\text{-}MHC^+$ cells in asthmatic mice were significantly higher than those in the control mice. The result indicates that SPCs in the OVA-induced asthmatic mice was increased. The mechanism of SPCs elevation in the peripheral mononuclear cells of asthmatic mice is unknown.

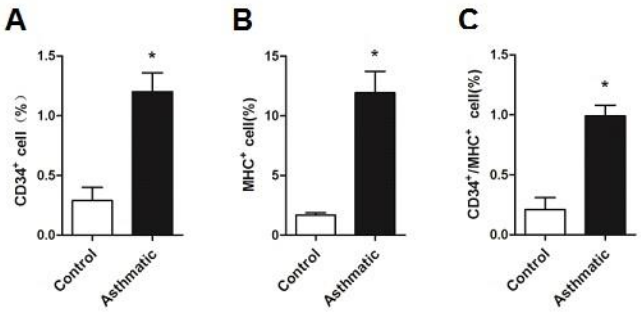

Figure 4. The flow cytometric analysis for $CD34^+$, MHC^+ and $CD34^+/MHC^+$ cells in the mononuclear cells of the asthmatic and control groups.*P<0.05 compared to control groups. Date presented as mean ± S.E.M of 20 animals.

We speculate that under the stimulation of specific inflammatory factors in asthma, SPCs in the bone marrow are vigorously proliferated and differentiated, which results in an increase of SPCs in the peripheral mononuclear cells. Although it was unclear how SPCs mobilize and integrate into the vessels and other targeted tissues, scholars have generally reasoned that chemotactic colonization of SPCs is similar to the infiltration of leukocytes into inflammatory tissues.[31] Therefore, the abnormally increased SPCs in the peripheral mononuclear cells of asthmatic mice are likely to be involved in the airway remodeling process of chronic asthma.

There were some limitations of our study, because the mechanism by which SPCs are increased in the peripheral mononuclear cells of asthmatic mice is unknown and it is also unclear which marker is more specific for SPCs. These questions need to be addressed in future studies. In this study, in order to determine the role of SPCs in asthma with airway remodelling, we just evaluated histological changes and SPCs levels, but the role of SPCs in the development of airway remodeling is also worth studying.

In summary, this study showed that the numbers of SPCs among the mononuclear cells of asthmatic mice were significantly higher than those in the control mice, indicating that SPCs have a definate relationship with the status of asthma. These results support our hypothesis that SPCs are involved in chronic airway remodeling. In addition, these results provide a necessary basis for further investigations of the role of SPCs in airway remodeling in asthma.

Authors' contributions

Dongyun Qin designed the research. Yongfu Wu and Hui Fu performed this research and analyzed the

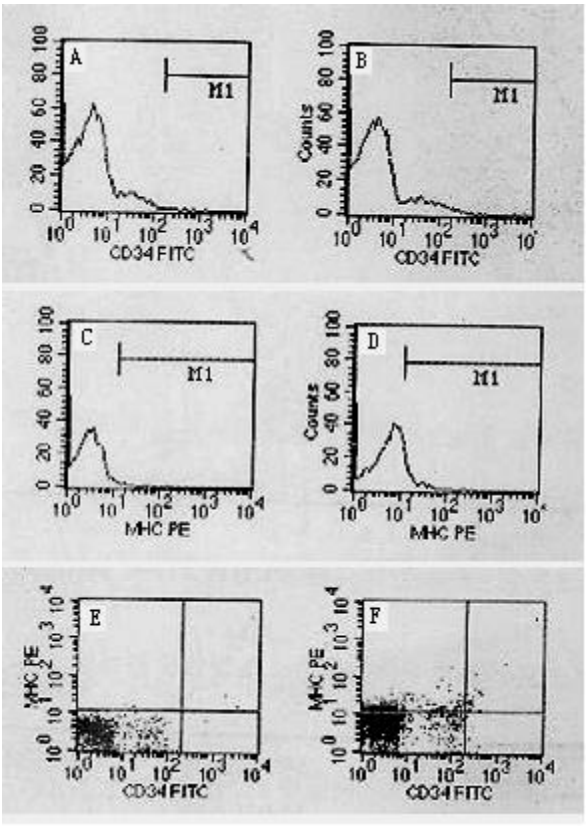

Figure 5. Expression of CD34 and SM-MHC on the surface of peripheral mononuclear cells in the asthmatic and control mice groups was detected by flow cytometry. The results for control mice are shown in panels A, C and E. The results for asthmatic mice are shown in panels B, D and F. A and B indicate the differential expression of CD34. C and D indicate the differential expression of SM-MHC. E and F indicate the differential expression of both CD34 and SM-MHC.

data. Huiling Yang, Hongqiang Wang, Hanbin Zhang contributed to the experiments. Dongyun Qin, Hui Fu and Yongfu Wu interpreted the data and wrote the manuscript. Dongyun Qin supervised all of the work. All of the authors have read and approved the final manuscript.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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