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Brg1 aggravates airway inflammation in asthma via inhibition of the PI3K/Akt/mTOR pathway

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ABSTRACT

The PI3K/Akt/mTOR pathway is thought to be closely associated with airway inflammation and is regulated by various upstream proteins. Brahma-related gene 1 (Brg1) plays an important role in chromatin remodeling and facilitates recruitment of essential transcription factors, leading to regulation of gene expression. Thus, the present study aimed at evaluating the anti-inflammatory role of Brg1 on house dust mite (HDM)-induced asthma through regulating the PI3K/Akt/mTOR pathway. The Brg1^{fl/fl} mice were crossbred with the SFTPC-Cre mice to generate bronchial epithelial cell specific Brg1 knockout mice, and LY294002 was used to inhibit PI3K. Western blot, immunofluorescence, immunoprecipitation, and immunohistochemical staining were used to detect the expression of proteins. An increase in Brg1 and a decrease in the PI3K/Akt/mTOR pathway activity were detected in asthmatic mice, but not in control mice. When Brg1 was knocked out, the asthma severity was ameliorated and the PI3K/Akt/mTOR pathway was activated. However, this protective effect could be suppressed by LY294002. Additionally, we observed that Brg1 was co-localized and co-immunoprecipitated with PI3K, using immunofluorescence and immunoprecipitation assays. Our results suggest that Brg1 might play an essential role in maintaining airway inflammation and affect the PI3K/Akt/mTOR pathway in asthma.

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1. Introduction

Asthma, a chronic airway inflammatory disease, affects more than 300 million asthmatic patients worldwide and seriously endangers human health [1]. In recent years, the prevalence rate of asthma in China has been increasing year after year [2]. Due to the complexity of etiology and pathogenesis, clinical treatment of asthma is also facing great challenges. Recent studies suggest that epigenetic regulation (heritable changes in gene expression that occur in the absence of alterations in DNA sequences) may in part mediate the complex gene-environment interactions that can lead to asthma [3]. Findings from multiple epidemiological and experimental studies indicate that asthma risk may be modified by epigenetic regulation [3]. Therefore, it is interesting to study the pathogenesis of asthma from the perspective of epigenetics. Brahma-related gene 1 (Brg1), a chromatin remodeling protein,

encoded by the SMARCA4 gene, is the core component of the mammalian switch/sucrose non-fermentable (SWI/SNF) class of chromatin remodeling complex, with ATPase activity. Brg1 has key roles in the pathogenesis of a wide range of human diseases by shaping the landscape of gene expression [4]. Brg1 increased transcription of proinflammatory genes in renal ischemia [5]. Inhibition of Brg1 increased the expression of cyclin-dependent kinase inhibitors and promoted cardiac myocyte regeneration [6]. In human colorectal carcinoma cells, knockdown of Brg1 down-regulated cyclin D1 expression level via inhibition of the PI3K/Akt pathway [7]. Therefore, we examined the role of Brg1 on downstream signaling in asthma. The phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of the rapamycin (mTOR) pathway is widely induced in asthma and affects airway inflammation in asthma. PI3K, a classical upstream kinase in the mTOR pathway, has been implicated in various immune responses and inflammatory processes. The kinase Akt is the main intermediate between PI3K and mTOR kinase [8]. Numerous studies on asthma pathogenesis indicate that PI3K/Akt modulates airway hyper-responsiveness (AHR), inflammation, and vascular permeability [9,10]. Blocking

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the PI3K/Akt signaling pathway could attenuate the early stages of airway remodeling in asthma by regulating the abnormal process of epithelial-mesenchymal transition and aggravating airway inflammation in asthma [11]. mTOR is a highly conserved serine/threonine kinase belonging to the family of phosphoinositide 3-kinase (PI3K)-like kinases, which has been implicated in various diseases, including cancer and type 2 diabetes [12,13]. Additionally, the mTORC2/Akt pathway is reported to be activated by calpain to regulate asthmatic airway smooth muscle remodeling [14]. Thus, several lines of evidence suggest the role of PI3K/Akt/mTOR pathway in the pathogenesis of asthma.

In our previous study, we reported that Brg1 inhibits E-cadherin expression in lung epithelial cells and disrupts epithelial integrity [15]. This is the first evidence that indicates the important role of the epigenetic molecule Brg1 in asthma. Since Brg1 may affect the PI3K signaling pathway, which is important in the pathogenesis of asthma, in this study, we investigated whether Brg1 can affect pathology of asthma via the PI3K/Akt/mTOR pathway.

2. Materials and methods

2.1. Animals

Female C57BL/6 mice (4–6 weeks old) were purchased from the Experimental Animal Center of Chongqing Medical University (Chongqing, China). AE2 and cubic cell specific Brg1-knockout (SFTPC-Cre/Brg1^{fl/fl}) mice were generated by hybridizing SFTPC-rtTA/(TetO)₇ transgenic mice (The Jackson Laboratory, CA, USA) with Brg1^{loxP/loxP} mice (kindly provided by Prof. Yujun Shi, Medical College of Sichuan University). Brg1 deletion in SFTPC-Cre/Brg1^{fl/fl} mice was achieved by administering doxycycline (1 mg/ml) in drinking water containing 0.4% sucrose. The various control mice were Brg1^{fl/fl} mice (no Cre) that received doxycycline. Experimental mice were housed under specific pathogen-free conditions and subjected to a 12 h/12 h dark/light cycle. The study was approved by the Ethics Committee of Chongqing Medical University.

2.2. Grouping and administration

A total of 16 female C57BL/6 mice were divided into control and asthma group. The mice of asthma group underwent house dust mite (HDM) challenge according to previous studies [16,17]. Briefly, mice received either 20 µg HDM (Greer, Los Angeles, CA, USA) dissolved in 30 µl normal saline (NS) (asthma group) or same volume of NS (control group) by nasal inhalation on days 0, 14, 21, 23, 25, 27, and 29. A total of 32 control/Brg1^{fl/fl} mice were divided into control/Brg1^{fl/fl} + NS and control/Brg1^{fl/fl} + LY294002 groups, and a total of 32 SFTPC-Cre/Brg1^{fl/fl} mice were divided into SFTPC-Cre/Brg1^{fl/fl} + NS and SFTPC-Cre/Brg1^{fl/fl} + LY294002 groups. These mice underwent HDM challenge and were subjected to intraperitoneal injection 0.5 mg/kg PI3K inhibitor (LY294002; MCE, New Jersey, USA) or NS. All mice were sacrificed at 24 h after the last challenge and then euthanized by intraperitoneal injection of 4% pentobarbital (0.5 mg/kg).

2.3. Measurement of airway hyper-responsiveness (AHR)

AHR was determined using an invasive pulmonary function instrument (EMKA Technologies, Paris, France) within 24 h after the final HDM challenge. Mice were anesthetized with 2% pentobarbital sodium, intubated, and placed in whole-body plethysmography chambers. Airway resistance and compliance were measured every second. Baseline and changes in airway resistance were determined using NS and methacholine (3.125, 6.25, 12.5, 25, or 50 mg/ml) respectively, which were nebulized at a volume of 20 µl

[16]. Lung resistance at each concentration was calculated.

2.4. Histological and immunohistochemical assessment of lung tissues

Left lung tissues were fixed in 4% formalin buffer and embedded in paraffin. The paraffin blocks were then serially sectioned into 4-µm-thick slices and subjected to immunohistochemical (IHC) staining. Antibody against Brg1 (1:200; Abcam, Massachusetts, USA) was used for IHC staining. Tissues or cells that were stained brown were recorded as being positive for Brg1. The intensity of IHC staining was quantified using Image-Pro Plus 6.0 software. For histochemical analysis, paraffin blocks were serially sectioned into 4-µm-thick slices and subjected to hematoxylin/eosin (H&E) or periodic acid-Schiff (PAS) (Leagene Biotechnology Co. Ltd, Beijing, China) staining. Three individual areas per section and eight lung tissues per group were evaluated for inflammatory cell infiltration.

2.5. Broncho alveolar lavage fluid (BALF) analysis and cell counting

The right lung of each animal was washed three times with 0.5 ml phosphate-buffered saline in order to collect BALF. Approximately 1.3 ml of the instilled fluid was consistently recovered. All the cells in BALF were collected by centrifugation and treated with red blood lysis buffer, after which they were counted by microscopy using a cell counter. The remaining inflammatory cells were smeared on slides, dried, and stained using Wright-Giemsa stain (Jiancheng Techno Co, Nanjing, China), according to the manufacturer's instructions to determine differential cell counts in accordance with conventional morphological criteria. At least 200 cells per slide were evaluated to obtain the differential cell counts.

2.6. Immunofluorescence detection

The expressions of Brg1 and PI3K in bronchial epithelial cells were determined by immunofluorescence staining. Lungs were excised and 'snap frozen' with liquid nitrogen and stored at -80 °C. Frozen sections (0.8 µm) were stained with primary antibodies, rabbit anti-Brg1 (1:50, Abcam, UK) and goat anti-PI3K (1:25, Abcam, UK) for 12 h at 4 °C. The sections were washed three times with PBS and stained for 30 min at room temperature with donkey anti-goat Cy3 and donkey anti-rabbit Cy5 secondary antibodies (1:500, Invitrogen, USA). Nuclei were counterstained with DAPI (4',6'-diamidino-2-phenylindole) (Invitrogen, USA) and fluorescence of the samples were captured using confocal laser scanning microscope (A1pR, Nikon, Tokyo, Japan).

2.7. Western blot and co-immunoprecipitation (co-IP) assay

Total protein from the right lung tissue was isolated using a total protein extraction kit (KeyGen BioTECH, China). Protein concentration was determined using the bicinchoninic acid (BCA) assay following the standard protocol. Protein samples (30 µg) were resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were then blocked and incubated overnight with the primary antibodies and subsequently incubated with horseradish peroxidase-linked goat anti-rabbit IgG secondary antibody (1:5000; Proteintech, China). The primary antibodies included mouse anti-Brg1 (1:300; Abcam, USA), rabbit anti-PI3K (1:500, Proteintech, China), rabbit anti-phospho-PI3K (1:500; CST, USA), rabbit anti-Akt (1:500, Proteintech, China), rabbit anti-phospho-Akt (1:500; CST, USA), rabbit anti-mTOR (1:500, Proteintech, China), rabbit anti-phospho-mTOR (1:500; Abcam, UK) and rabbit anti-β-actin (1:1000; Proteintech, China).

Immunoreactivity was visualized by an enhanced chemiluminescence (ECL) kit (ThermoFisher, USA). Band intensities were quantified using Quantity One software (version 4.6.2; Bio-Rad, Hercules, CA, USA) and normalized to β -actin. The co-IP assay [6] was performed using Protein A/G agarose (Beyotime, China), following the manufacturer's instructions and normal rabbit anti-IgG (Abcam, UK) was used as a control antibody. Harvested samples containing input (protein pretreated with nothing), IgG (protein pre-treated with A/G agarose and rabbit anti-IgG), anti-PI3K (protein pre-treated with A/G agarose and rabbit anti-PI3K), and anti-Brg1 (protein pre-treated with A/G agarose and rabbit anti-Brg1) were analyzed by western blotting.

2.8. Statistical analyses

Data were expressed as mean $\pm$ standard error of the mean. GraphPad Prism software (version 5.0; GraphPad, San Diego, CA, USA) was used for statistical analyses. Two-way analysis of variance was conducted to determine statistically significant differences in the tested variables among the different groups. If an overall test was significant, Tukey's test was used for specific comparisons between groups. $P < 0.05$ were considered statistically significant. All experiments were repeated at least thrice with consistent outcomes.

3. Results

3.1. High expression of Brg1 in HDM-induced asthmatic mice

C57BL/6 mice were subjected to intranasal injection of HDM to establish an asthma mouse model (Fig. 1A). The Brg1 protein levels in lung tissues were detected by western blot and

immunohistochemistry. Immunohistochemical detection showed that Brg1 expression in asthma group was higher than that in the control group (Fig. 1B, $P < 0.05$) and were further confirmed using western blotting (Fig. 1C and D).

3.2. Brg1 co-immunoprecipitated with PI3K and decreased PI3K/Akt/mTOR signaling pathway in HDM induced asthmatic mice

The PI3K/Akt/mTOR pathway is very important for airway epithelial cell repair in asthma. We explored potential role of Brg1 in this pathway in asthmatic mice. Immunofluorescence of wild-type asthmatic mouse lung tissue showed Brg1 and PI3K co-expression in bronchial epithelial cells (Fig. 2A), which was further supported by the co-IP assay that showed Brg1 interaction with PI3K (Fig. 2B). Subsequently, the expression of PI3K, Akt, and mTOR proteins were detected by western blotting. The ratios of p-PI3K/PI3K, p-Akt/Akt, and p-mTOR/mTOR were significantly decreased in asthmatic mice compared with control mice (Fig. 2C and D, $P < 0.05$).

3.3. Brg1 knockout in asthmatic mice increased the activation of the PI3K/Akt/mTOR pathway

In order to study the effect of Brg1 on PI3K/Akt/mTOR pathway in asthma, we used mice with specific knockout of Brg1 in the airway epithelial cells (Fig. 3A). Western blot assay showed that Brg1 expression in lung tissue of SFTPC-Cre/Brg^{fl/fl} mice was less than control/Brg^{fl/fl} mice group (Fig. 3B and C, $P < 0.05$). Then we established asthma models in Brg^{fl/fl} mice (Fig. 3D). To further explore the molecular mechanism of Brg1, we treated SFTPC-Cre/Brg^{fl/fl} asthmatic mice with the PI3K inhibitor-LY294002. Western blot analysis showed that the ratios of p-PI3K/PI3K, p-Akt/Akt, and

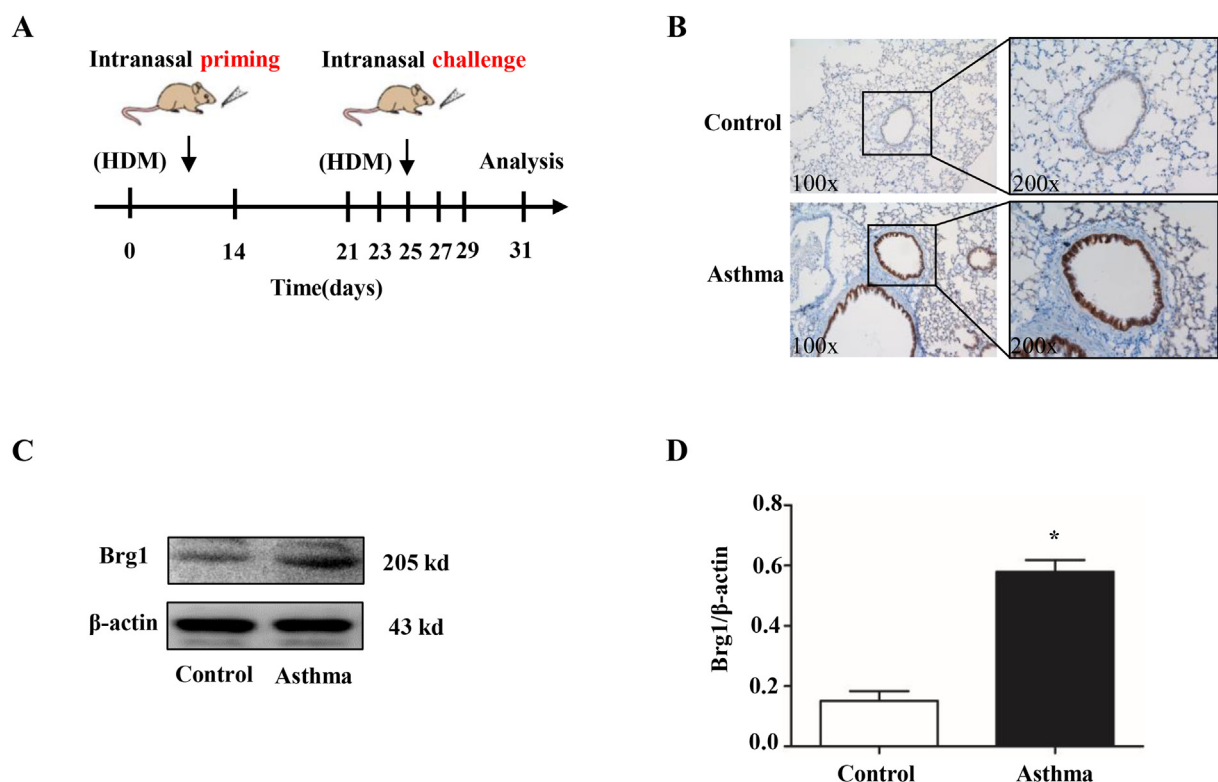


Fig. 1. An increased expression of Brg1 in HDM-induced asthmatic mice. (A) Protocol for inducing asthmatic mice. (B) Immunohistochemical detection of Brg1 in the lung tissues of control and asthmatic mice; brown staining represents positive expression, the pictures are 100 $\times$ and 200 $\times$ respectively. (C) Representative western blots showing the expression of Brg1 in the lungs of control and asthma group. (D) The intensity of Brg1 is normalized to β -actin ($n = 8$ per group; t -test; * $P < 0.05$ vs. control group).

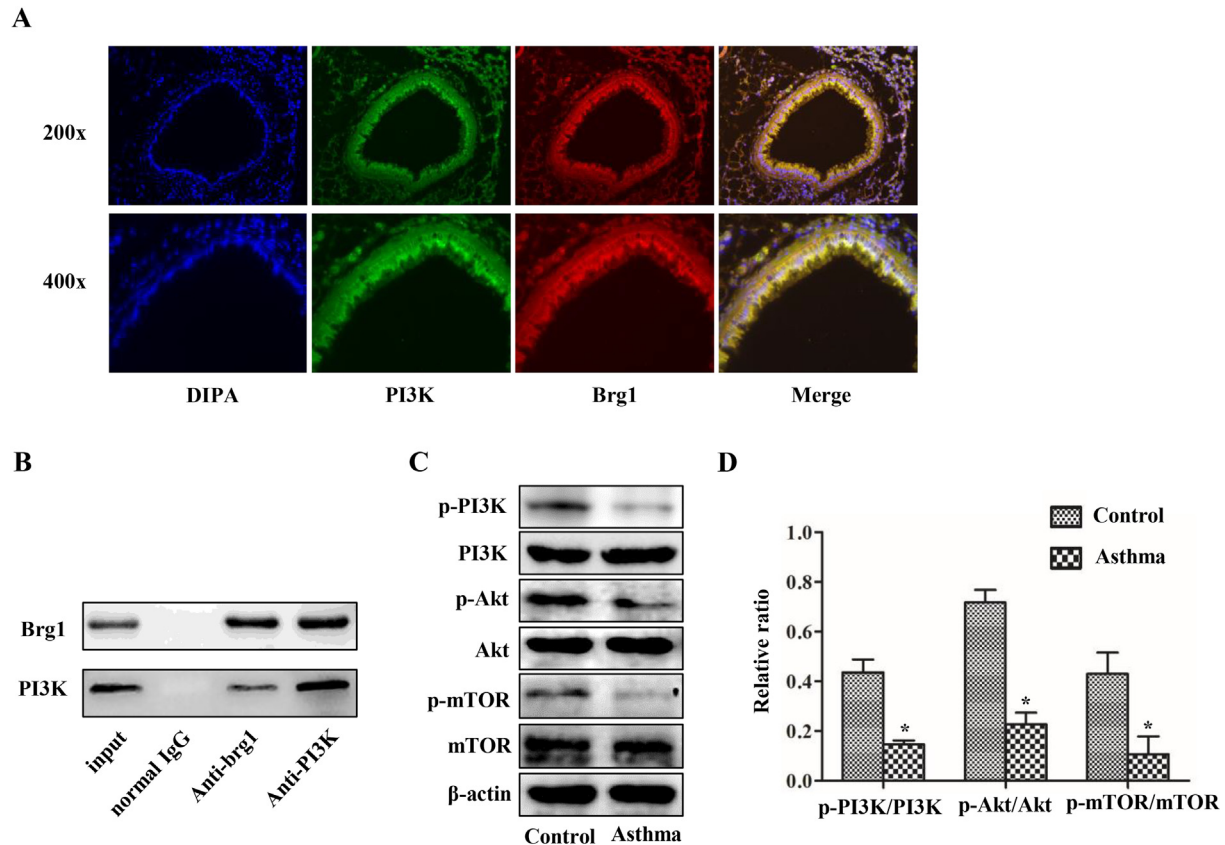


Fig. 2. Brg1 interacted with PI3K and inhibited the activation of PI3K/Akt/mTOR pathway. (A) Immunofluorescence detection of Brg1 and PI3K in lung tissues of asthmatic mice, Brg1 (red), PI3K (green), DAPI (blue). (B) Co-immunoprecipitation of Brg1 for lung tissues from asthma mice indicated Brg1 interacted with PI3K. (C) The representative western blots show the expressions of PI3K/Akt/mTOR in the lungs of control group and asthma group. (D) The phospho-PI3K/total PI3K, phospho-Akt/total Akt and phospho-mTOR/total mTOR statistical graph ($n = 8$ per group; t -test; * $P < 0.05$ vs. control group). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

p-mTOR/mTOR were increased in SFTPC-Cre/Brg^{fl/fl} + NS group compared with control/Brg^{fl/fl} + NS group and were decreased in SFTPC-Cre/Brg^{fl/fl} + LY294002 group when compared with SFTPC-Cre/Brg^{fl/fl} + NS group (Fig. 3E and F).

3.4. PI3K inhibitor abrogated the effect of Brg1 on airway inflammation in asthmatic mice

We observed that Brg1 was highly expressed in asthmatic mice and inhibited the PI3K/Akt/mTOR pathway in asthmatic mice. In order to confirm whether Brg1 promoted pathogenesis of asthma via the PI3K pathway, we treated Brg^{fl/fl} mice with PI3K inhibitor-LY294002. The results of pulmonary function detection displayed a decrease in lung resistance of SFTPC-Cre/Brg^{fl/fl} + NS group compared with control/Brg^{fl/fl} + NS, whereas it was increased in the SFTPC-Cre/Brg^{fl/fl} + LY294002 group (Fig. 4A, $P < 0.05$). The numbers of eosinophils and lymphocytes in the BALF of SFTPC-Cre/Brg^{fl/fl} + LY294002 group were higher than in the SFTPC-Cre/Brg^{fl/fl} + NS group (Fig. 4B, $P < 0.05$). Histological analysis using H&E and PAS staining showed that infiltration of inflammatory cells around the bronchus and airway mucus secretion were rescued by LY294002 in SFTPC-Cre/Brg^{fl/fl}+NS group mice (Fig. 4C).

4. Discussion

Asthma is characterized by persistent chronic airway inflammation, which leads to mucus hypersecretion and airway hyper-responsiveness [18]. We investigated the involvement of Brg1, a

chromatin-remodeling protein, in the regulation of airway inflammation, focusing on the role of Brg1 on the PI3K/Akt/mTOR pathway. Our previous data demonstrated that Brg1 was highly expressed in asthmatic patients and Brg1 knockout decreased airway epithelial integrity in ovalbumin-induced asthmatic mice [15]. In this study, we found that the Brg1 expression in HDM-induced asthmatic mice was higher than in the control group, indicating an important role of Brg1 in asthma airway inflammation. Notably, Chaiyachati et al. [19], previously demonstrated that Brg1 acted in part via remodeling-inflammation related factors to sensitize Tregs to inflammatory cues, suggesting a potential role for Brg1 in the regulation of asthma airway inflammation. Particularly, Brg1 is distinctly involved in the chromatin remodeling required for activating pro-inflammatory genes in a temporal manner [20]. Moreover, over-expression of Brg1 significantly down-regulated four important inflammation factors, including MMP3, TIMP2, COX2, and IL6 in MH7A cells [21]. A few studies have found that Brg1 is not only related to inflammation, but also closely related to cell proliferation and repair. In a study of cardiac endothelial injury, Fang et al., demonstrated that Brg1 and Brm integrated various proinflammatory cues leading to adhesion molecule trans-activation and endothelial malfunction [22]. In liver diseases, Brg1 could promote liver regeneration by epigenetically activating the Wnt/ β -catenin pathway in mice [23]. For the treatment of lung cancer, Brg1 positively regulated the expression of miR-148b, leading to inhibition of cell proliferation, metastasis, restraint of epithelial-mesenchymal transition, and inactivation of the Wnt/ β -catenin signaling pathway [24]. In Brg1 knockdown lung cancer

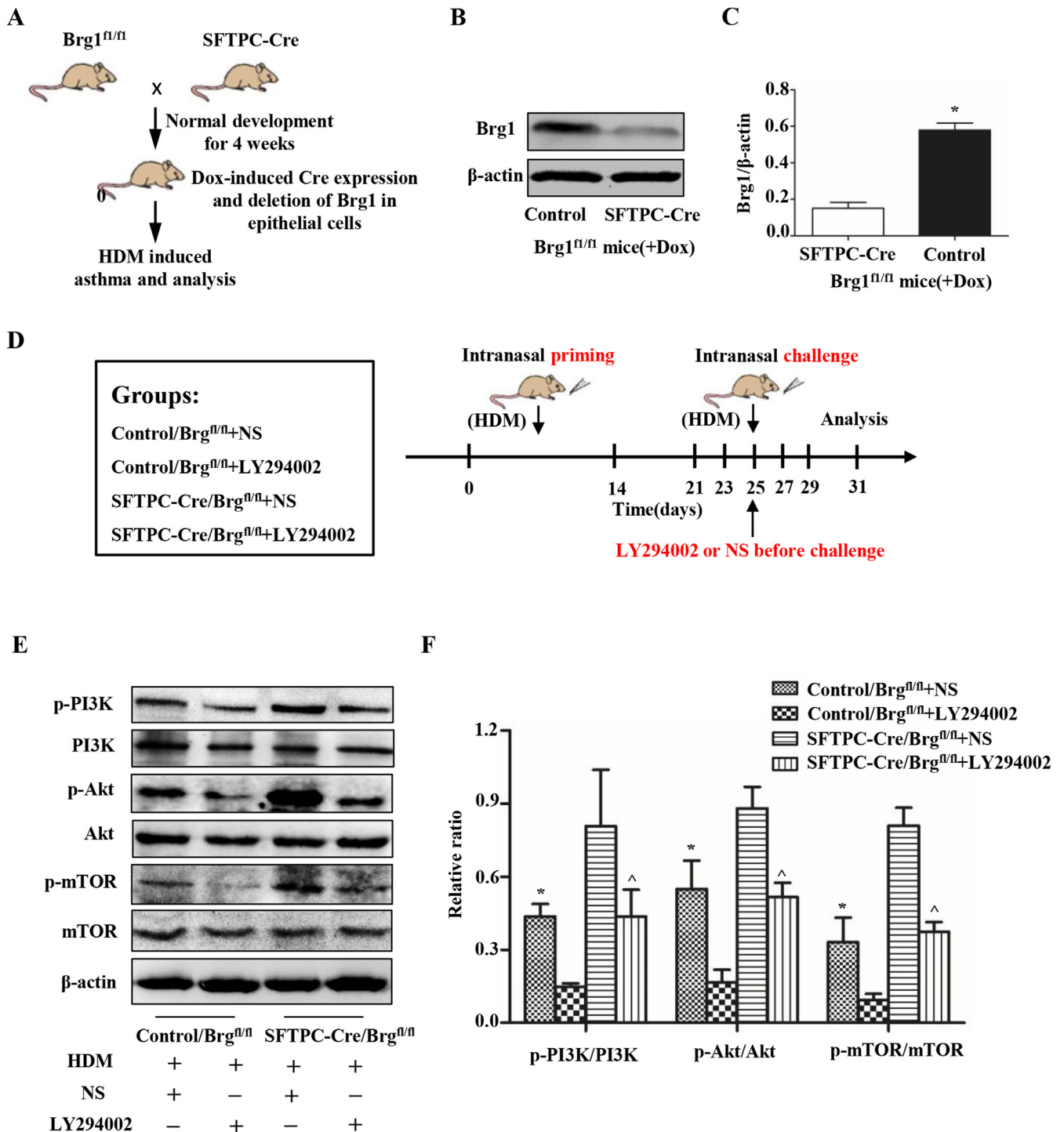


Fig. 3. Mice lacking Brg1 in airway epithelial cells show increased activation of PI3K/Akt/mTOR pathway and is rescued by PI3K inhibitor. (A) Schematic for generating SFTPC-Cre/Brg1^{fl/fl} mice and Dox-induced Brg1 deletion in airway epithelial cells. (B) Western blot of Brg1 expression of control/Brg1^{fl/fl} group and SFTPC-Cre/Brg1^{fl/fl} group. (C) Statistical graph of Brg1 in the lungs of control/Brg1^{fl/fl} group and SFTPC-Cre/Brg1^{fl/fl} group. (D) Representative western blots showing the expression of Brg1 in the lungs of control/Brg1^{fl/fl} and SFTPC-Cre/Brg1^{fl/fl} group. (E) Western blot analysis for phospho-PI3K, total PI3K, phospho-Akt, total Akt, phospho-mTOR and total mTOR in control/Brg1^{fl/fl} + NS, control/Brg1^{fl/fl} + LY294002 group, SFTPC-Cre/Brg1^{fl/fl} + LY294002 and SFTPC-Cre/Brg1^{fl/fl} + NS group (F) Statistical graph of western blot results (n = 8 per group; t-test; *P < 0.01, ^P < 0.01 vs. SFTPC-Cre/Brg1^{fl/fl} + NS group).

cells the number and the size of tumors remained unchanged compared with the controls [25]. These evidences indicated that Brg1 could promote inflammation and inhibit cell proliferation and repair function in different diseases. The pathological process of asthma involves airway inflammation and repair of airway

epithelial injury. Our findings demonstrate high expression of Brg1 in HDM-induced asthmatic mice and decreased airway inflammation, airway hyper-responsiveness and mucus hypersecretion in Brg1 knockout of mouse bronchial epithelial cells on the other. These observations strongly indicate that the role of Brg1 is crucial

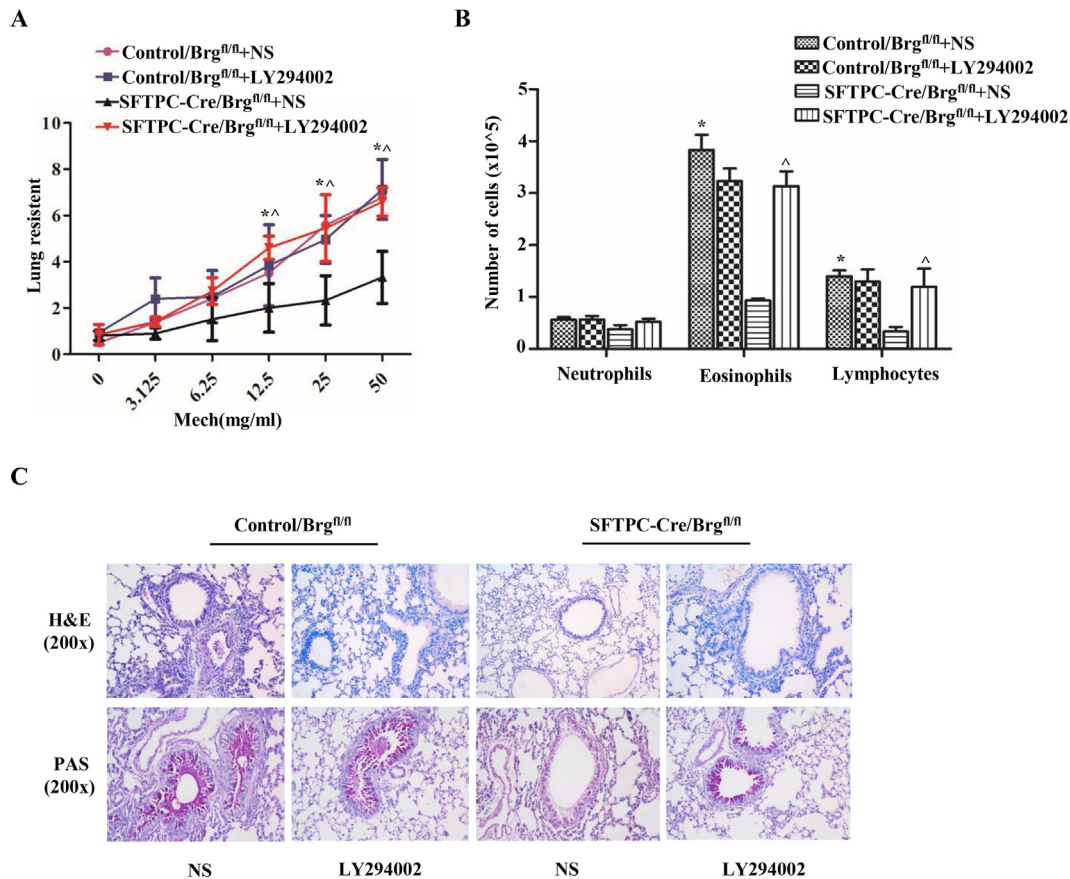


Fig. 4. Alleviative airway inflammation in Brg1-deficient mice is restored by PI3K inhibitor. (A) Lung resistance in control/Brg^{fl/fl} or in SFTPC-Cre/Brg^{fl/fl} mice. (B) Eosinophils, neutrophils and lymphocytes in the BALF. (C) Representative hematoxylin and eosin (H&E) and periodic acid Schiff (PAS) staining of lung sections from control/Brg^{fl/fl} or SFTPC-Cre/Brg^{fl/fl} mice. (n = 8 per group; t-test; *P < 0.01, ^P < 0.01 vs. SFTPC-Cre/Brg^{fl/fl} + NS group).

for airway inflammation and repair of airway epithelial injury in asthma. We, therefore, concluded that Brg1 participates in the regulation of asthma airway inflammation processes and the specific mechanism needs to be further explored.

The PI3K/Akt/mTOR pathway is hyperactivated or altered in many diseases and regulates a broad range of cellular processes including survival, proliferation, growth, metabolism, angiogenesis, and metastasis, which are also important in asthma. Many reports have demonstrated an important role of the PI3K/Akt/mTOR pathway in asthma. In our present study, the PI3K/Akt/mTOR pathway was inhibited in asthmatic mice with high expression of Brg1, and knockout of Brg1 in bronchial epithelial cells of asthmatic mice promoted PI3K/Akt/mTOR pathway expression. A recent study illustrated that patients experiencing an asthma attack, when compared with patients in asthma remission, showed significantly elevated serum mTOR levels [26]. Another study by T. Liu et al., showed that particulate matter 2.5 (PM_{2.5}) could induce autophagy via inhibition of the PI3K/Akt/mTOR signaling pathway in human bronchial epithelial cells [27]. Supporting this contention, the mTOR inhibitor rapamycin has been reported to block airway inflammation in HDM-sensitized mice, mediated by the reduced expression of Th2- and Th17-type cytokines [28]. It was found that inhibiting the PI3K/Akt/mTOR pathway in lung cancer cells could inhibit the proliferation of tumor cells [29]. In proliferative vitreoretinopathy (PVR), PI3K/AKT/mTOR signaling pathway inhibitors effectively inhibited proliferation of retinal pigment epithelial cells [30]. mTOR signaling also plays an important role in airway epithelial cell proliferation. mTORC1-related changes in the

proliferation, size and survival of epithelial cells may contribute to hypertrophy and remodeling of the airway wall in asthma [31,32]. Treatment of HDM-induced asthma with an mTOR inhibitor augmented airway inflammatory responses, as indicated by significant increases in the number of BALF eosinophils, lymphocytes and neutrophils [33]. These studies revealed that the PI3K/AKT/mTOR signaling pathway maybe related to the regulation of airway inflammation and repair of airway epithelial injury in asthma. In our study, we found that the PI3K/AKT/mTOR signaling pathway was inhibited in asthmatic mice whereas Brg1 was highly expressed in asthma lung tissues. Meanwhile, knockout of Brg1 in asthmatic mice increased activation of the PI3K/AKT/mTOR pathway. Therefore, we speculated that the high expression of Brg1 in asthma may promote airway inflammation and reduce the repair of airway epithelial injury via inhibiting the PI3K/Akt/mTOR signaling pathway. In order to confirm this speculation, we used a specific Brg1 airway epithelial cell knockout mouse model and a PI3K inhibitor. Our data indicate that knockout of Brg1 could reduce airway inflammation in asthmatic mice, while using a PI3K inhibitor in knockout mice increased airway inflammation. These results suggested that Brg1 promoted airway inflammation in asthma partly by inhibiting the PI3K/Akt/mTOR pathway. Although our study does not discriminate the specific mechanism of the Brg1 and PI3K interaction, we plan to conduct further in vitro studies in the future to demonstrate the mechanism.

In summary, the present findings demonstrate that Brg1 is highly expressed in HDM-induced asthma and aggravates airway inflammation partly via inhibiting the PI3K/Akt/mTOR pathway.

The role of Brg1 on the PI3K/Akt/mTOR pathway may be a desirable target for developing therapeutic strategies aimed at the treatment of asthma.

Conflicts of interest

The authors declare no conflict of interest.

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Transparency document

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