



Chrysin alleviates allergic inflammation and airway remodeling in a murine model of chronic asthma

Jing Yao^a, Mingzi Jiang^b, Yunshi Zhang^a, Xing Liu^a, Qiang Du^{a,*}, Ganzhu Feng^{a,*}

^a Department of Respiratory Medicine, the Second Affiliated Hospital of Nanjing Medical University, 121 Jiangjiayuan Road, Nanjing, Jiangsu 210000, PR China

^b Department of Respiratory Medicine, The First People's Hospital of Kunshan, Qianjinxi Road No. 91, Kunshan, Jiangsu 215300, PR China

ARTICLE INFO

Article history:

Received 23 August 2015

Received in revised form 4 January 2016

Accepted 5 January 2016

Available online 15 January 2016

Keywords:

Asthma

Chrysin

Airway hyperresponsiveness

Inflammation

Airway remodeling

ABSTRACT

Asthma is a chronic airway inflammatory disorder and progresses mainly due to airway remodeling. Chrysin, a natural flavonoid, has been reported to possess multiple biologic activities, including anti-inflammation, anti-oxidation and anti-proliferation. The present study aimed to investigate whether chrysin could relieve allergic airway inflammation and remodeling in a murine model of chronic asthma and the mechanism involved. The female BALB/c mice sensitized and challenged with ovalbumin (OVA) successfully developed airway hyperresponsiveness (AHR), inflammation and remodeling. The experimental data showed that chrysin could alleviate OVA-induced AHR. Chrysin could also reduce OVA-induced increases in the number of inflammatory cells, especially eosinophils, interleukin (IL) -4, and IL-13 in bronchoalveolar lavage fluid (BALF) and total IgE in serum. The decreased interferon- γ (IFN- γ) level in BALF was also upregulated by chrysin. In addition, inflammatory cell infiltration, goblet cell hyperplasia and the expression of α -smooth muscle actin (α -SMA) around bronchioles were suppressed by chrysin. Furthermore, the phosphorylation levels of Akt and extracellular signal-regulated kinase (ERK) could be decreased by chrysin, which are associated with airway smooth muscle cell (ASMC) proliferation. These results indicate the promising therapeutic effect of chrysin on chronic asthma, especially the progression of airway remodeling.

© 2016 Elsevier B.V. All rights reserved.

1. Introduction

Asthma is a chronic airway inflammatory disease associated with reversible airway obstruction, airway hyperresponsiveness (AHR) and airway remodeling [1]. T-helper type 1 (Th1)/T-helper type 2 (Th2) imbalance is classically considered to lead to asthma, related to up-regulated Th2 cytokine production, decreased Th1 cytokine production, increased IgE and eosinophilic inflammation in the airway [2]. Airway inflammation and remodeling are believed to interact with each other, resulting in the occurrence and progress of asthma. Among these, airway remodeling manifests a range of structural alterations, including increased deposition of extracellular matrix proteins (ECMs), goblet cell metaplasia, angiogenesis and increased airway smooth muscle mass, which has been proposed to contribute to persistent airflow limitation and lower baseline of lung function [3]. Airway smooth muscle cells (ASMCs), as a pivotal element involved in asthma, not only participates in airway inflammation and AHR, but also play a key role in remodeling

mainly due to its increased mass. Several signaling molecules are involved in ASMC proliferation, such as extracellular signal-regulated kinase (ERK) and Akt [4]. Nowadays, bronchodilatation and anti-inflammation are the main therapies for asthma [5], which can't stop the progression of structural changes effectively [6]. Corticosteroids still play a crucial role in asthma control, but with lots of side effects [7]. New effective drugs are urgently needed for asthma therapy.

An increasing number of researches indicate that natural products and traditional medicines may be promising in asthma treatment. Flavonoids are commonly present in vegetables, nuts, fruits, beverages or herbal remedies, described as a health-promoting and disease-preventing dietary supplement [8]. Chrysin (5, 7-dihydroxyflavone) is a natural flavonoid, widely distributed in medicinal herbs [9,10]. It has been confirmed that chrysin exerts multiple biological activities, such as anti-inflammation, anti-proliferation and anti-oxidation [11–13]. However, the impacts of chrysin on chronic asthma are still unknown. In our previous study, we have demonstrated that chrysin could significantly inhibit the proliferation of human airway smooth muscle cells (HASMCs) through ERK1/2 pathway [14]. Therefore, we assumed that chrysin might play a therapeutic role in chronic asthma. In the present study, we aimed to define the impacts of chrysin on the ovalbumin (OVA)-sensitized and challenged chronic asthma model and the mechanism involved. We also used dexamethasone (DEX) as a positive treatment control [15].

* Corresponding authors at: Department of Respiratory Medicine, The Second Affiliated Hospital of Nanjing Medical University, 121 Jiangjiayuan Road, Nanjing, Jiangsu 210011, PR China.

E-mail addresses: jingshuoyue@163.com (Q. Du), fenggz@njmu.edu.cn (G. Feng).

¹ The two corresponding authors contributed equally to this article.

2. Method

2.1. Antigen sensitization, challenge and treatment

Fifty-eight female specific pathogen-free (SPF) BALB/c mice (6 weeks old) were purchased from Cavens Laboratory Animal Company (Changzhou, China) and housed in the SPF facility. The mice were acclimatized for a week before experimental study. All experimental procedures were approved by the institutional animal ethics committee. Animal experiments were carried out in strict accordance with the Nanjing Medical University's guidelines for their care and use. Mice were randomly divided into four groups: (a) control group; (b) OVA group; (c) DEX group and (d) chrysin group. On Days 1 and 14, the mice in the OVA, DEX and chrysin groups were immunized by intraperitoneal injection of 20 µg OVA (Grade V; Sigma, St Louis, MO, USA) emulsified in 2.25 mg aluminum hydroxide (InvivoGen, San Diego, CA, USA) in a total volume of 0.2 ml. For aerosolized challenges, mice were placed in a Plexiglas box (29 × 22 × 18 cm) and exposed to aerosolized OVA or phosphate buffer solution (PBS) using an ultrasonic nebulizer (PAPI TurboBOY N, Germany). From Day 21, the mice of OVA, DEX and chrysin groups were exposed to aerosolized 1% OVA for 30 min per day, 3 days per week for 8 weeks. The mice in the DEX group received an intraperitoneal injection of DEX (2 mg/kg body weight) 30 min before every OVA challenge. Chrysin (purity 97%; Sigma, St. Louis, MO, USA) was suspended in a 1% carboxymethylcellulose (CMC) solution. From Day 21, the mice in chrysin group were administered with chrysin (100 mg/kg body weight) intragastrically and daily for 8 consecutive weeks. The mice in the control group received the same schedule for sensitization and challenge with PBS. After sensitization and challenge, six animals of each group were used for detection of airway resistance, the other six were used for collection of bronchoalveolar lavage fluid (BALF) and lung tissues (as shown in Fig. 1).

2.2. Evaluation of AHR

Airway responsiveness to acetylcholine chloride (Ach; Sigma, St. Louis, MO, USA) was measured with a whole-body and invasive plethysmography (Buxco Electronics Inc., NY, USA). Twenty-four hours after the last OVA challenge, mice were anesthetized by intraperitoneal injection of pentobarbital sodium (75 mg/kg body weight). A tracheal tube was inserted into the trachea via a tracheotomy. Then, mice were placed in the whole-body plethysmography chamber and the tracheal tube was connected to the ventilator for mechanical

ventilation with a tidal volume of 0.2 ml and a frequency of 140 breaths/min. After 3 min of equilibration on the ventilator, PBS and increasing doses of Ach (3.125, 6.25, 12.5 and 25 mg/ml) were administered through the ventilator with an ultra-sonic nebulizer. Lung resistance (R_L) was measured to express the change of AHR.

2.3. Analysis of BALF and serum

The mice were anesthetized and bled from the retro-orbital venous plexus 24 h after the last OVA challenge. Blood samples were centrifuged (20 min, 4 °C, 1000 rpm), and the plasma was stored at −80 °C until use. After the removal of blood, the lungs were washed two times by intratracheal instillation of 0.5 ml ice-cold PBS via a tracheotomy to collect BALF. The BALF was centrifuged (10 min, 4 °C, 1000 rpm) and the supernatants were transferred and stored at −80 °C before use for the cytokine detections. Cell pellets were resuspended in ice-cold PBS with a final volume of 30 µl. The numbers of total and different inflammatory cells in BALF were determined with a hemocytometer. The levels of interleukin (IL)-4, interferon-γ (IFN-γ) (Biotechnology Company, Wuhan, China) and IL-13 (eBioscience, San Diego, Calif., USA) in BALF and total serum IgE (CUSABIO Biotechnology Company, Wuhan, China) were determined by ELISA according to the manufacturer's protocol.

2.4. Histological examination

The right lower lungs obtained from sacrificed mice were immersed in 4% paraformaldehyde overnight, and then embedded in paraffin and sectioned. The sections (5 µm thick) were stained with hematoxylin-eosin (HE), periodic acid-Schiff (PAS) and Masson's trichrome stain [16]. Histological analysis was performed by pathologists blinded to grouping. To quantify the level of mucus production in airways, numbers of PAS-negative and PAS-positive epithelial cells (goblet cells) in individual bronchioles were counted. Results were expressed as the percentage of PAS-positive cells per bronchiole, which was calculated from the number of PAS-positive epithelial cells per bronchiole divided by the total number of epithelial cells in each bronchiole. The area of peribronchial trichrome staining in lung tissues was quantified with an image-analysis system (Image-Pro Plus 6.0; Media Cybernetics, Inc., Georgia, USA). Results are expressed as the area of trichrome staining per µm length of basement membrane of bronchioles with an internal diameter of 150–200 µm by counting at least 10 bronchioles in each slide.

2.5. Immunohistochemistry analysis

Sections of lung tissues were deparaffinized in xylene and rehydrated in graduated ethanol solutions. Endogenous peroxidase activity was quenched by 3% H₂O₂. To avoid non-specific absorption of immunoglobulin, specimens were blocked with 5% non-fat milk. Thereafter, specimens were incubated with mouse monoclonal antibody against α-smooth muscle actin (α-SMA; DAKO, Glostrup, Denmark) at a dilution of 1:50 at 4 °C overnight. Substitution of the primary antibody with normal mouse IgG was used as a negative control. Sections were then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Bioworld Technology, Shanghai, China) for 30 min at room temperature, followed by staining with diaminobenzidine (DAB; Santa Cruz, CA, USA) [17,18]. Sections were counter-stained with hematoxylin and observed under a light microscope. Data were expressed as the area of α-SMA immunostaining per µm length of basement membrane of bronchioles with an internal diameter of 150–200 µm by counting at least 10 bronchioles in each slide, which was examined using an image-analysis system (Image-Pro Plus 6.0; Media Cybernetics, Inc., Georgia, USA).

Chronic asthma murine model

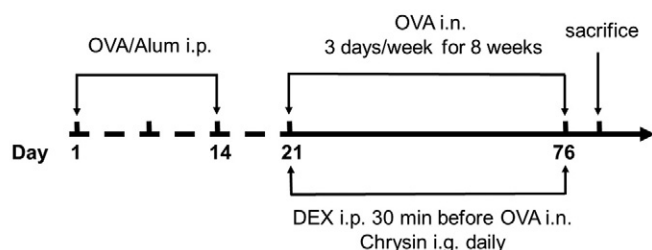


Fig. 1. Experimental protocol for chronic asthma murine model. BALB/c mice were sensitized with OVA and aluminum hydroxide by intraperitoneal injection on Days 1 and 14 and then challenged with aerosolized 1% OVA three times per week for 8 weeks from Day 21. The control mice were sensitized and challenged with PBS using the same protocol. DEX was administered via intraperitoneal injection 30 min before the challenge. Chrysin was administered intragastrically and daily from Day 21. Mice were sacrificed within 24 h after the last OVA challenge and treatment. OVA, ovalbumin; Alum, aluminum hydroxide; i.p., intraperitoneal injection; i.g., intragastric administration; i.n., inhalation; DEX, dexamethasone.

2.6. Western blot analysis

The left lung tissues were homogenized and lysed. Thereafter, tissue homogenates were centrifuged (5 min, 4 °C, 10,000 rpm) and the supernatants were collected as the total protein extracts. The protein concentrations were determined with the method of bicinchoninic acid [19]. Equivalent amounts of protein were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred onto a polyvinylidene difluoride membrane [20]. The membrane was blocked with 5% dried milk for 1 h at room temperature and then exposed to monoclonal antibody against β -actin (Santa Cruz, CA, USA), ERK (Bioworld Technology, St. Louis Park, MN, USA), p-ERK (Cell Signaling Technology, Beverly, MA, USA), Akt (Affinity Biosciences, Cincinnati, OH, USA) or p-Akt (Affinity Biosciences, Cincinnati, OH, USA) overnight at 4 °C with gentle shaking. The membrane was incubated with horseradish peroxidase-conjugated secondary antibody (1:1000) for 90 min at room temperature. Immunoreactive bands were detected by the ECL reagents. The intensity of bands was quantified using Image Lab Version 4.0 software (Bio Rad Laboratories). β -actin was used as an internal control for protein loading.

2.7. Statistical analysis

Data were expressed as mean $\pm$ SEM. Statistical analysis was carried out using one-way analysis of variance (ANOVA) among three groups or more and Student's t-test between two groups with SPSS software (SPSS 19, Chicago, IL, USA). Values of $p < 0.05$ were considered statistically significant.

3. Result

3.1. The effect of chrysin on airway hyperresponsiveness to acetylcholine chloride

Lung resistance (R_L) was used to estimate the effect of chrysin on airway responsiveness to increasing concentrations of Ach using an invasive whole-body plethysmography. As illustrated in Fig. 2, there was no significant difference between groups at the baseline of airway resistance. R_L increased in a dose-dependent way by administration of Ach in each group except the control one in which there was only a

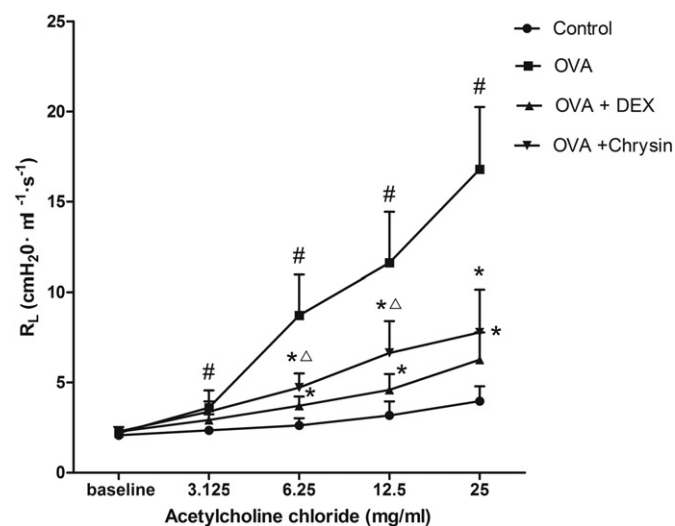


Fig. 2. The effect of chrysin on airway hyperresponsiveness to acetylcholine chloride. Effect of chrysin on AHR in response to increasing concentrations of aerosolized acetylcholine chloride (3.125, 6.25, 12.5, and 25 mg/ml) or PBS 24 h after the last OVA challenge in mechanically ventilated mice. Airway responsiveness was assessed by lung resistance (R_L). The values presented are mean $\pm$ SEM (# $p < 0.05$ vs. control group; * $p < 0.05$ vs. OVA group, $\Delta p < 0.05$ vs. DEX group, $n = 6$).

slight increase. R_L was increased in OVA-challenged mice compared with that of the control group (vs. control group, $p < 0.05$). This up-regulation of R_L was reversed by both DEX and chrysin (vs. OVA group, $p < 0.05$). There was no significant difference between DEX and chrysin groups except at 6.25 mg/ml and 12.5 mg/ml (chrysin group vs. DEX group, $p < 0.05$).

3.2. The effect of chrysin on airway inflammation

To evaluate the airway inflammation, inflammatory cells recruited in BALF were differentially counted. The levels of inflammatory cells, including total cells, macrophages, eosinophils, lymphocytes and neutrophils, were significantly elevated in the OVA group versus the control group, as shown in Fig. 3 (vs. control group, $p < 0.05$). Both DEX and chrysin treatment significantly reduced the numbers of total inflammatory cells, macrophages, eosinophils, lymphocytes and neutrophils (vs. OVA group; $p < 0.05$). However, DEX has reduced eosinophils at a greater degree compared with chrysin (chrysin group vs. DEX group, $p < 0.05$).

To further confirm the anti-inflammatory property of chrysin, histopathological examinations were performed. The marked inflammatory cell infiltration into peribronchial and perivascular lesions in the lung tissue was observed in the OVA group compared with the control group using HE staining. Treatment with DEX or chrysin reduced the inflammatory cell infiltration compared with the OVA group (Fig. 4).

3.3. The effect of chrysin on the release of Th1/Th2 cytokines in BALF and total IgE level in serum

As shown in Fig. 5A and B, OVA sensitization and challenge induced the elevation of Th2 cytokines, both IL-4 and IL-13 in the BALF (vs. control group, $p < 0.05$). Treatment with either DEX or chrysin significantly decreased the concentrations of IL-4 and IL-13 (vs. OVA group; $p < 0.05$). In contrast, IFN- γ level decreased in OVA treated group (vs. control group, $p < 0.05$) and was upregulated by both DEX and chrysin (vs. OVA group; $p < 0.05$, Fig. 5C). There is no significance between DEX and chrysin groups on the levels of these three cytokines.

The level of serum total IgE in the OVA group was significantly increased by OVA sensitization and challenge compared to that of the control group, as shown in Fig. 5D (vs. control group, $p < 0.05$). However, a significant reduction of serum total IgE was observed in mice treated with DEX and chrysin (vs. OVA group; $p < 0.05$). No significant difference was detected between DEX and chrysin groups.

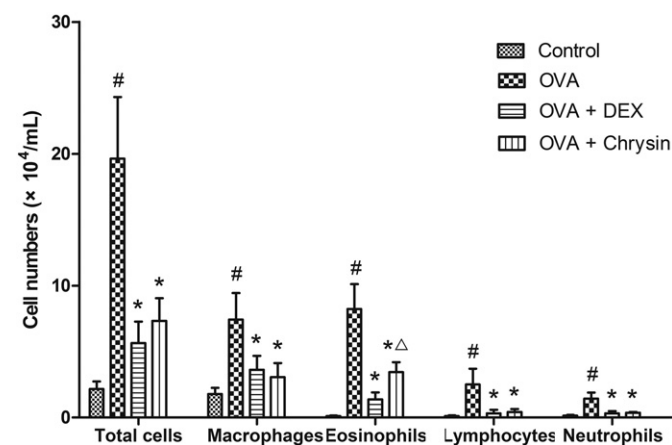


Fig. 3. The effect of chrysin on airway inflammatory cells in BALF. The numbers of total and different inflammatory cells in BALF were evaluated using a hemocytometer. The values presented are mean $\pm$ SEM (# $p < 0.05$ vs. control group; * $p < 0.05$ vs. OVA group, $\Delta p < 0.05$ vs. DEX group, $n = 6$).

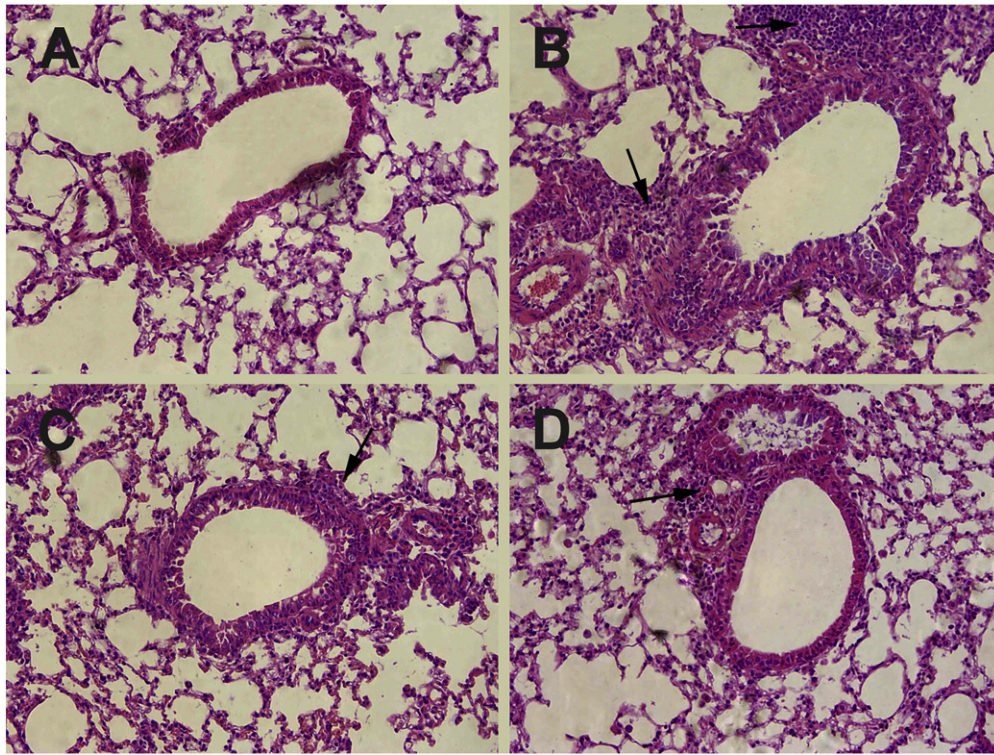


Fig. 4. Chrysin attenuates airway inflammation induced with OVA. Histological examination of the airway inflammation is shown with HE staining (magnification $\times 200$). A: control group; B: OVA group; C: DEX group; D: chrysin group. Black arrows indicate the inflammatory cells in HE stained lung tissue.

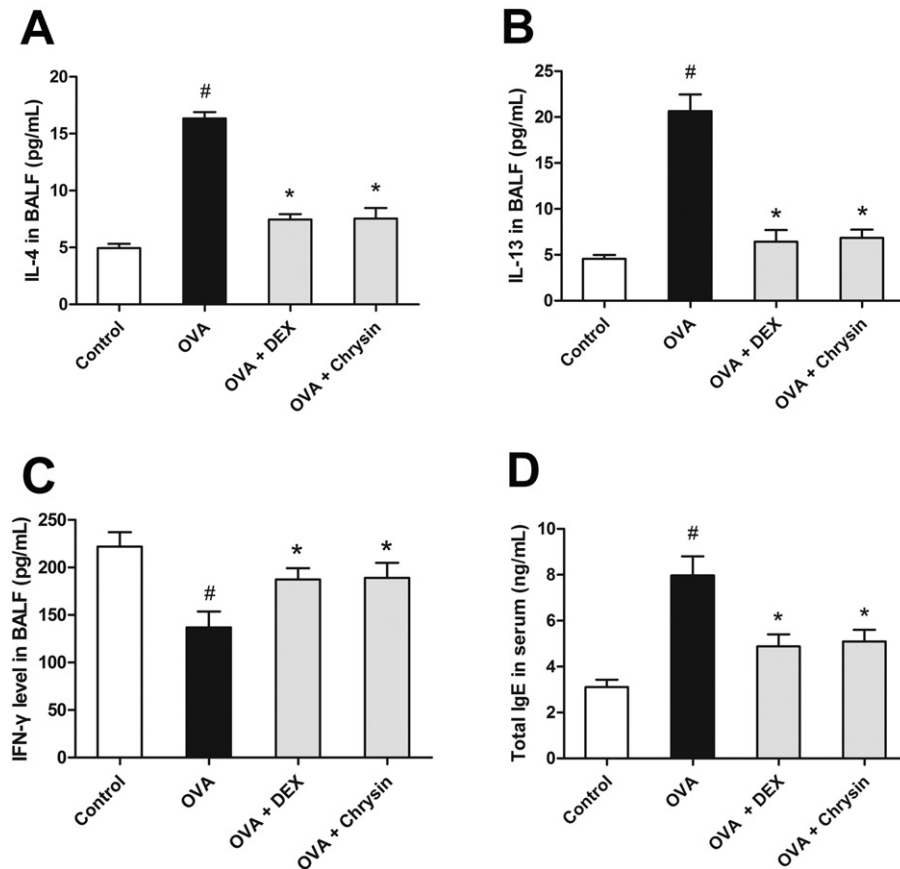


Fig. 5. The effect of chrysin on Th1/Th2 cytokines in BALF and serum total IgE level. BALF and serum were collected 24 h after the last OVA challenge. Levels of IL-4 (A), IL-13 (B), INF- γ (C) and serum total IgE (D) were analyzed using ELISAs. The values presented are mean $\pm$ SEM ([#] $p < 0.05$ vs. control group; ^{*} $p < 0.05$ vs. OVA group, $n = 6$).

3.4. The effect of chrysin on airway remodeling

Goblet cell hyperplasia, extracellular matrix (collagen) deposition and increased airway smooth muscle mass are key elements contributing to the pathologic changes of airway remodeling and constantly used to evaluate the severity of airway remodeling in asthma. In the present study, PAS staining, Masson's trichrome, and immunohistochemistry were applied respectively to estimate goblet cell hyperplasia and mucus hypersecretion, extracellular matrix deposition and pulmonary α -SMA protein expression.

Overproduction of mucus and hyperplasia of goblet cells were observed in the bronchial airways of mice in the OVA group (Fig. 6A, B, E, vs. control group, $p < 0.05$). Compared with the OVA group, DEX and chrysin significantly alleviated mucus secretion and goblet cell hyperplasia (Fig. 6B, C, D, E, vs. OVA group, $p < 0.05$). There was no significant difference between the effects of these two drugs.

Collagen overdeposition was detected around the bronchial airways of mice in the OVA group (Fig. 7A, B, E, vs. control group, $p < 0.05$). Compared with the OVA group, DEX and chrysin treatment significantly alleviated collagen deposition to a similar degree (Fig. 7B, C, D, E, vs. OVA group, $p < 0.05$).

Airway smooth muscle remodeling was expressed as pulmonary α -SMA protein expression. As shown in Fig. 8A–E, the peribronchial α -SMA staining (per μm of bronchi basement membrane) in the OVA group mice were significantly increased compared to the baseline levels of that in the control group (vs. control group, $p < 0.05$). The treatment of both DEX and chrysin could decrease α -SMA protein expression in lung tissues in the murine model of chronic asthma (vs. OVA group, $p < 0.05$). There was no significant difference between the two groups.

3.5. The effect of chrysin on Akt and ERK1/2 signaling pathway activation

In our present study, Western blot analysis revealed that chrysin could modify the phosphorylation of Akt and ERK1/2. As shown in Fig. 9, OVA sensitization and challenge could increase the phosphorylation levels of both Akt and ERK1/2 (vs. control group, $^*p < 0.05$). Either DEX or chrysin could significantly inhibit the up-regulated phosphorylation of Akt and ERK1/2 (vs. OVA group; $^*p < 0.05$). There is no significant difference between the roles of these two agents. Any intervention has little effect on the total levels of Akt and ERK1/2.

4. Discussion

Chrysin, a natural flavonoid, has been reported to attenuate allergic airway inflammation, the immediate and late-phase asthmatic responses to aerosolized-OVA exposure in an acute animal model of asthma [21,22]. However, asthma behaves as a chronic disease in human beings. In our present study, to investigate the effect of chrysin on chronic asthma, we used OVA to induce its pathogenic process in a murine model and the typical histopathological changes were successfully established. Chronic OVA inhalation induced a dramatic increase of airway AHR, Th1/Th2 imbalance, inflammatory cells infiltrating into the peribronchial tissues, goblet cells hyperplasia, excessive collagen deposition and increased airway smooth muscle mass around bronchia. Chrysin intervention could significantly attenuate both airway inflammation and structural changes in the lung tissues, predicting that chrysin might be a promising agent for the treatment of chronic asthma, especially airway remodeling.

Numerous studies have proved that inflammation is a central element in asthma. The asthmatic airway shows inflammatory cell infiltration, consisting of a mixture of T lymphocytes and eosinophils [23, 24]. Among them, eosinophils are increased especially in allergic disease. Eosinophils recruited into the peribronchioles mediate the pathogenesis of asthma, including AHR and airway remodeling [25, 26]. Furthermore, recent data suggest that eosinophils can also contribute to airway remodeling via inducing ASMC proliferation [27]. Our experimental result showed that chrysin reduced the number of inflammatory cells leaked into BALF, especially eosinophils. This effect was also affirmed by morphological changes in lung tissues evaluated by HE staining, which revealed that chrysin decreased the inflammatory cell infiltration around the bronchioles and vessels. These results suggest that chrysin could suppress airway inflammation, and the inhibition of eosinophil infiltration may be part of the mechanism of chrysin's effect on remodeling, with ASMC as the target cell.

Asthma is classically considered as a Th2-associated disorder due to Th1/Th2 imbalance. Most asthmatic patients are caused by common inhaled allergens. These allergens presented by antigen presenting cells (APCs) stimulate Th2 cell proliferation and subsequently result in the production and release of Th2 cytokines, such as IL-4 and IL-13 [28]. IL-4 can promote initial differentiation of Th2 cell, B cell activation, IgE synthesis and eosinophil recruitment [29–31]. IL-13 is involved in allergic AHR, B cell activation and airway remodeling including goblet cell

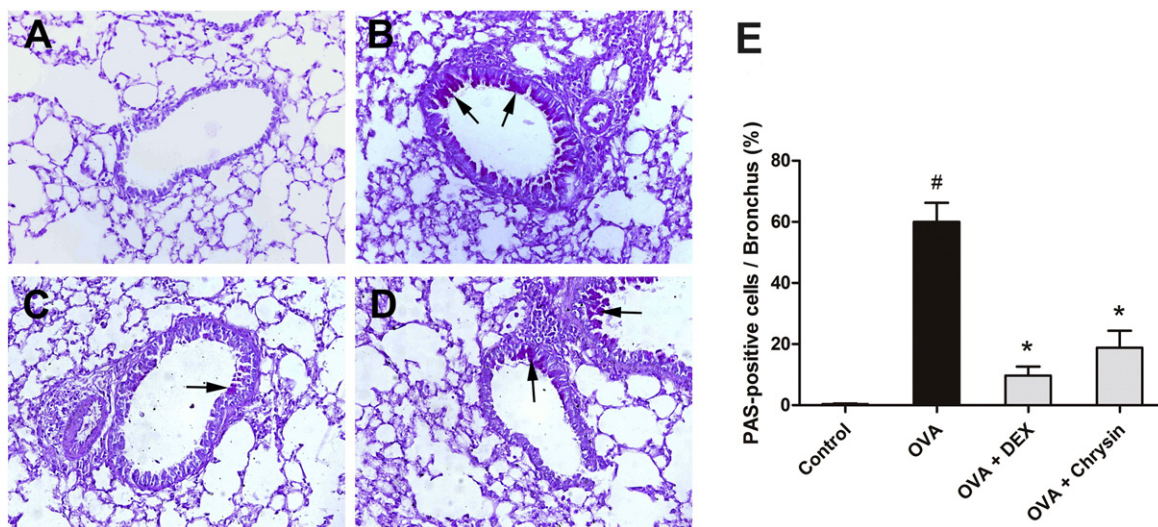


Fig. 6. Chrysin reduces goblet cell metaplasia. Goblet cell metaplasia is assessed with PAS staining (magnification $\times 200$). A: control group; B: OVA group; C: DEX group; D: chrysin group; E: a bar graph expressed by the percentage of PAS-positive cells per bronchiole. The values presented are mean $\pm$ SEM ($^*p < 0.05$ vs. control group; $^*p < 0.05$ vs. OVA group, $n = 6$). Black arrows indicate goblet cell.

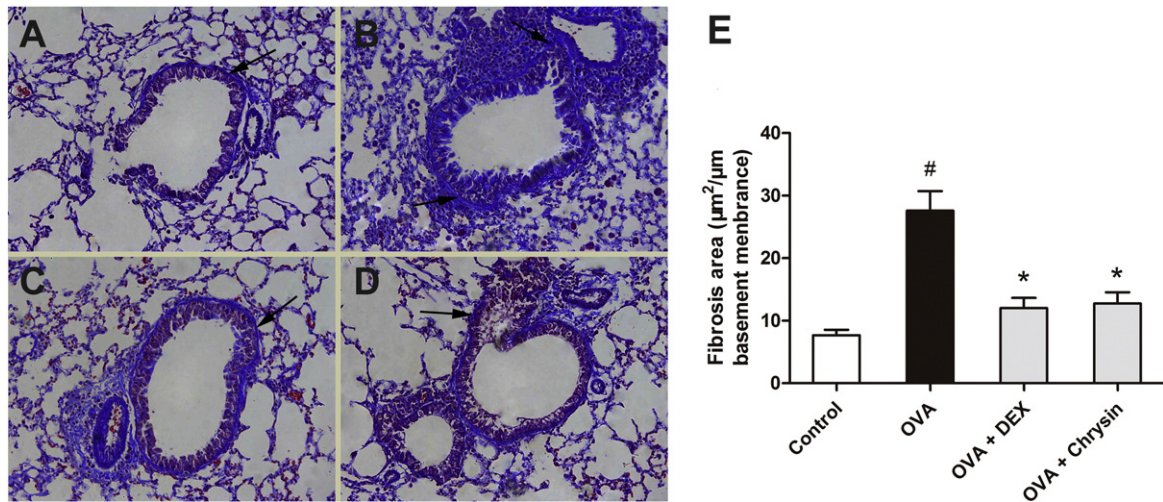


Fig. 7. Chrysin suppressed collagen deposition. Collagen deposition in lung tissue was examined by Masson's trichrome staining (magnification $\times 200$). A: control group; B: OVA group; C: OVA + DEX group; D: OVA + chrysin group; E: a bar graph expressed by the area of fibrosis per μm length of basement membrane of bronchioles. The values presented are mean $\pm$ SEM ([#] $p < 0.05$ vs. control group; ^{*} $p < 0.05$ vs. OVA group, $n = 6$). Black arrows indicate collagen accumulation.

metaplasia and inflammatory cell infiltration [31–33]. On the other hand, it has been reported that administration of IFN- γ , a Th1 cytokine, effectively attenuates allergy-induced airway inflammation and AHR in mice [34]. In our present study, chrysin reduced IL-4 and IL-13 levels in BALF, and IgE level in serum of asthmatic mice. Chrysin also upregulated IFN- γ level in BALF in asthmatic mice. Therefore, chrysin may attenuate the pathological change in asthma through mitigating the Th1/Th2 imbalance.

AHR is an important characteristic clinical feature of asthma, and many factors contribute to its development. Airway inflammation is considered as the key trigger of AHR. The activation of eosinophils is closely associated with AHR and AHR is absent in an animal model with a deficiency of eosinophils [35,36]. Airway structural changes, such as increased mass of airway smooth muscle (ASM), also lead to persistent AHR [37]. In addition, IL-4 and IL-13 can play a regulatory role in AHR [32]. In our research, the inhibition of AHR by chrysin is in accordance with its impacts on inflammatory cells, cytokines and ASM, further confirming the therapeutic effect of chrysin in chronic asthma.

Airway mucus hypersecretion, produced and secreted from goblet cells, is an important cause of airway obstruction, asthma deterioration and fatal asthma [38,39]. Goblet cell hyperplasia is one of the critical pathophysiologic changes in airway remodeling of asthma [40]. Besides, collagen deposition is also another crucial structural change of airway remodeling, contributing to irreversible airway limitation. In this study, chrysin decreased OVA-induced goblet cell hyperplasia and collagen deposition, indicating its potential suppression of airway remodeling directly.

The increased mass of smooth muscle cells around airways is associated with increasing disease severity of asthma and the decline of pulmonary function. Airway smooth muscle is involved in asthmatic inflammatory process by interacting with inflammatory cells and bronchial hyperresponsiveness due to its contractility. Moreover, ASM even exerts proinflammatory and immunomodulatory functions through its synthetic properties and the expression of a range of cell surface molecules [41]. It has been reported that ASM from asthmatic patients who died from acute exacerbation was much more increased

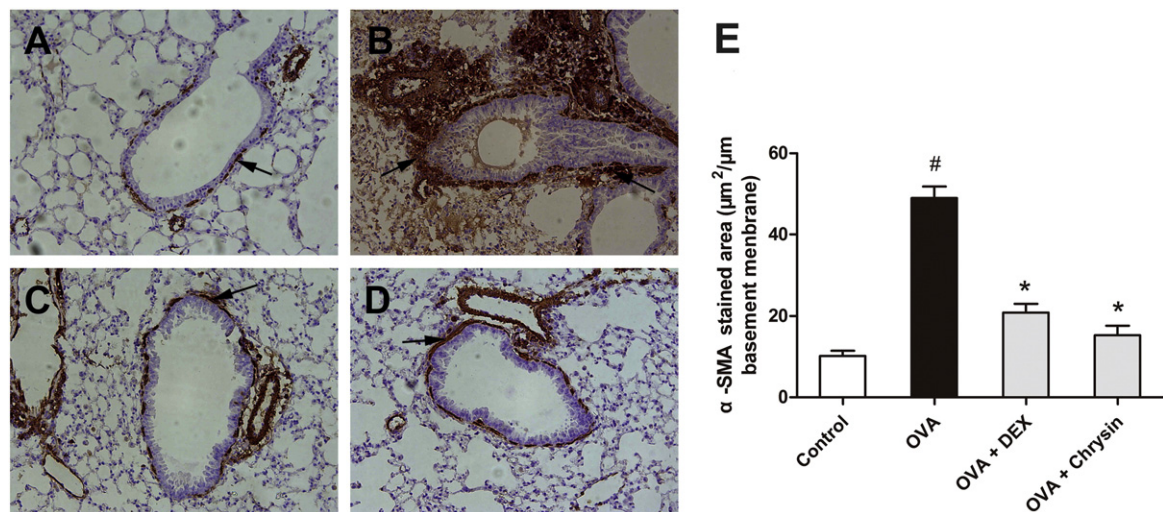


Fig. 8. Chrysin prevents airway smooth muscle hyperplasia. The expression of α -SMA in lung tissue was examined by immunohistochemistry (magnification $\times 200$). A: control group; B: OVA group; C: DEX group; D: chrysin group; E: a bar graph expressed by the area of α -SMA staining per μm length of basement membrane of bronchioles. The values presented are mean $\pm$ SEM ([#] $p < 0.05$ vs. control group; ^{*} $p < 0.05$ vs. OVA group, $n = 6$). Black arrows indicate α -SMA expression.

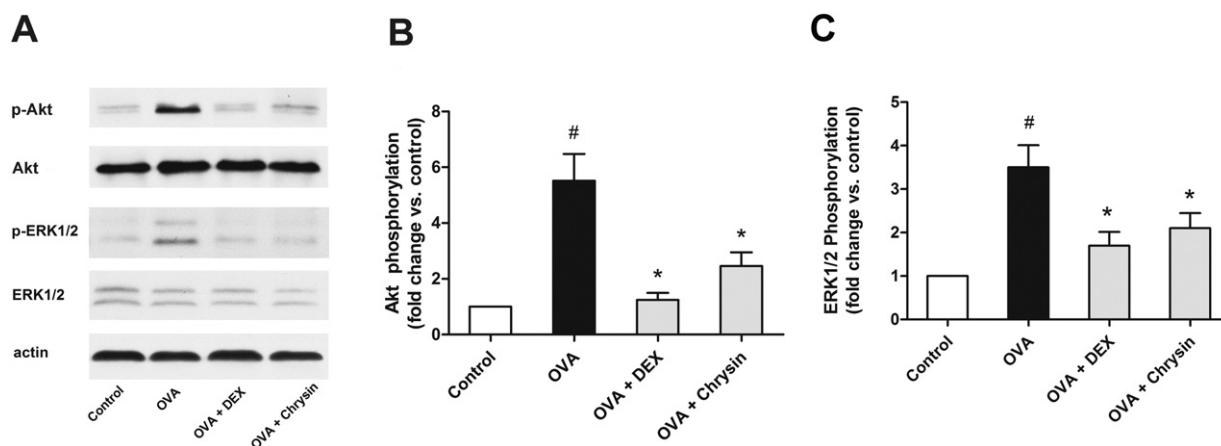


Fig. 9. Chrysin inhibits the phosphorylation of Akt and ERK1/2. The levels of Akt, p-Akt, ERK1/2 and p-ERK1/2 in protein extracts from lung tissues were analyzed with Western blot (A). The bar graph represents percent phosphorylation level compared with the control group (B and C). Densitometric data are expressed as mean $\pm$ SEM ([#] $p < 0.05$ vs. control group; ^{*} $p < 0.05$ vs. OVA group, $n = 6$).

than from those who died from another disease [42]. The mass of ASM increases even in patients with mild to moderate asthma [43], implying it plays a role in the early stage of asthma. α -SMA is widely viewed as a contractile element in ASMC. Increased levels of α -SMA and other contractile components serve to promote ASMC proliferation and migration, which are pivotal for airway remodeling [44]. In this investigation, changes in the expression of α -SMA were analyzed to examine the effect of chrysin on ASM. The reduction in α -SMA mediated by chrysin may be partly due to its impact on interactional cytokines and inflammatory cells and partly to its direct action on ASMC itself, which is also in line with chrysin's effect on HASMCs in vivo. This result further indicates chrysin's therapeutic function on airway remodeling.

The increased mass of ASM is mainly due to hyperplasia. Polypeptide growth factors, inflammatory mediators, cytokines as well as contractile agonists can stimulate ASM growth [45]. Despite those numerous mediators, only several critical downstream signaling pathways are converged on. ERK and Akt are crucial components in signal transduction associated with cell proliferation. Both of them are activated in ASMC and participate in the regulation of ASMC proliferation [46]. It has been reported that LY294002 (a phosphatidylinositol 3-kinase (PI3K) inhibitor) can suppress Akt phosphorylation and then inhibit ASMC proliferation [47]. Another Akt inhibitor, TAT-Akt-in, is shown to block IgE-induced ASMC proliferation [48]. Inhibition of ERK1/2 phosphorylation by PD184352 or PD98059 (the ERK phosphorylation inhibitors) could alleviate ASMC proliferation induced by chemokine [49,50]. Previous data certifies that chrysin could inhibit U937 proliferation and the phosphorylation of Akt in U937 [51]. Another study confirmed that chrysin could suppress cell proliferation and ERK signaling in B16-F1 and A375 cell [52]. In our previous investigation, we have demonstrated that chrysin could significantly inhibit the proliferation of human airway smooth muscle cells (HASMCs) through ERK1/2 pathway [14]. In the present study, the mechanism of chrysin's anti-proliferation is further investigated and the experimental results showed that chrysin may suppress ASMC proliferation through reducing the phosphorylation of both Akt and ERK1/2.

In conclusion, we are the first to demonstrate that chrysin could significantly improve chronic asthma in a murine model on multiple aspects, including AHR, eosinophilic inflammation, airway remodeling and Th2 cytokines. Moreover, chrysin has a comparable function to DEX on asthma. These results indicate the potential clinical benefits of chrysin in chronic asthma treatment.

Conflict of interest

The authors declare that no actual or potential conflict of interest exists in relation to this article.

Acknowledgments

This study was supported by the Natural Science Foundation of Jiangsu Province (BK20131436).

References

- [1] W. Manuyakorn, P.H. Howarth, S.T. Holgate, Airway remodelling in asthma and novel therapy, *Asian Pac. J. Allergy Immunol.* 31 (2013) 3–10 launched by the Allergy and Immunology Society of Thailand).
- [2] W.W. Busse, R.F. Lemanske Jr., Asthma, *N. Engl. J. Med.* 344 (2001) 350–362.
- [3] G. Chiappara, R. Gagliardo, A. Siena, M.R. Bonsignore, J. Bousquet, G. Bonsignore, et al., Airway remodelling in the pathogenesis of asthma, *Curr. Opin. Allergy Clin. Immunol.* 1 (2001) 85–93.
- [4] O. Tliba, R.A. Panettieri Jr., Noncontractile functions of airway smooth muscle cells in asthma, *Annu. Rev. Physiol.* 71 (2009) 509–535.
- [5] E.D. Bateman, S.S. Hurd, P.J. Barnes, J. Bousquet, J.M. Drazen, M. FitzGerald, et al., Global strategy for asthma management and prevention: GINA executive summary, *Eur. Respir. J.* 31 (2008) 143–178.
- [6] R. Berair, C.E. Brightling, Asthma therapy and its effect on airway remodelling, *Drugs* 74 (2014) 1345–1369.
- [7] E.O. Vianna, R.J. Martin, Bronchodilators and corticosteroids in the treatment of asthma, *Drugs Today* 34 (1998) 203–223.
- [8] Y.J. Moon, X. Wang, M.E. Morris, Dietary flavonoids: effects on xenobiotic and carcinogen metabolism, *Toxicol. in Vitro* 20 (2006) 187–210.
- [9] C.A. Williams, J.B. Harborne, M. Newman, J. Greenham, J. Eagles, Chrysin and other leaf exudate flavonoids in the genus *Pelargonium*, *Phytochemistry* 46 (1997) 1349–1353.
- [10] P. Rapt, V. Misik, A. Stasko, I. Vrabec, Redox intermediates of flavonoids and caffeic acid esters from propolis: an EPR spectroscopy and cyclic voltammetry study, *Free Radic. Biol. Med.* 18 (1995) 901–908.
- [11] H. Cho, C.W. Yun, W.K. Park, J.Y. Kong, K.S. Kim, Y. Park, et al., Modulation of the activity of pro-inflammatory enzymes, COX-2 and iNOS, by chrysin derivatives, *Pharmacol. Res.* 49 (2004) 37–43.
- [12] T. Phan, X.M. Yu, M. Kunnimalaiyaan, H. Chen, Antiproliferative effect of chrysin on anaplastic thyroid cancer, *J. Surg. Res.* 170 (2011) 84–88.
- [13] T. Lapidot, M.D. Walker, J. Kanner, Antioxidant and prooxidant effects of phenolics on pancreatic beta-cells in vitro, *J. Agric. Food Chem.* 50 (2002) 7220–7225.
- [14] J. Yao, Y.S. Zhang, G.Z. Feng, Q. Du, Chrysin inhibits human airway smooth muscle cells proliferation through the extracellular signal-regulated kinase 1/2 signaling pathway, *Mol. Med. Rep.* (2015).
- [15] R.K. Kumar, C. Herbert, P.S. Thomas, L. Wollin, R. Beume, M. Yang, et al., Inhibition of inflammation and remodeling by roflumilast and dexamethasone in murine chronic asthma, *J. Pharmacol. Exp. Ther.* 307 (2003) 349–355.
- [16] J. Pu, G.S. Mintz, S. Biro, J.B. Lee, S.T. Sum, S.P. Madden, et al., Insights into echo-attenuated plaques, echolucent plaques, and plaques with spotty calcification: novel findings from comparisons among intravascular ultrasound, near-infrared spectroscopy, and pathological histology in 2294 human coronary artery segments, *J. Am. Coll. Cardiol.* 63 (2014) 2220–2233.
- [17] T. Yao, X. Ying, Y. Zhao, A. Yuan, Q. He, H. Tong, et al., Vitamin D receptor activation protects against myocardial reperfusion injury through inhibition of apoptosis and modulation of autophagy, *Antioxid. Redox Signal.* 22 (2015) 633–650.
- [18] Q. He, J. Pu, A. Yuan, W.B. Lau, E. Gao, W.J. Koch, et al., Activation of liver-X-receptor alpha but not liver-X-receptor beta protects against myocardial ischemia/reperfusion injury, *Circ. Heart Fail.* 7 (2014) 1032–1041.
- [19] P. Shan, J. Pu, A. Yuan, L. Shen, L. Shen, D. Chai, et al., RXR agonists inhibit oxidative stress-induced apoptosis in H9c2 rat ventricular cells, *Biochem. Biophys. Res. Commun.* 375 (2008) 628–633.

- [20] J. Pu, A. Yuan, P. Shan, E. Gao, X. Wang, Y. Wang, et al., Cardiomyocyte-expressed farnesoid-X-receptor is a novel apoptosis mediator and contributes to myocardial ischaemia/reperfusion injury, *Eur. Heart J.* 34 (2013) 1834–1845.
- [21] Q. Du, X. Gu, J. Cai, M. Huang, M. Su, Chrysin attenuates allergic airway inflammation by modulating the transcription factors T-bet and GATA-3 in mice, *Mol. Med. Rep.* 6 (2012) 100–104.
- [22] J.Y. Lee, J.M. Kim, C.J. Kim, Flavones derived from nature attenuate the immediate and late-phase asthmatic responses to aerosolized-ovalbumin exposure in conscious guinea pigs, *Inflamm. Res.* 63 (2014) 53–60.
- [23] N. Carroll, C. Cooke, A. James, The distribution of eosinophils and lymphocytes in the large and small airways of asthmatics, *Eur. Respir. J.* 10 (1997) 292–300.
- [24] Q. Hamid, Y. Song, T.C. Kotsimbos, E. Minshall, T.R. Bai, R.G. Hegele, et al., Inflammation of small airways in asthma, *J. Allergy Clin. Immunol.* 100 (1997) 44–51.
- [25] A.B. Kay, The role of eosinophils in the pathogenesis of asthma, *Trends Mol. Med.* 11 (2005) 148–152.
- [26] A. Kanda, V. Driss, N. Hornez, M. Abdallah, T. Roumier, G. Abboud, et al., Eosinophil-derived IFN-gamma induces airway hyperresponsiveness and lung inflammation in the absence of lymphocytes, *J. Allergy Clin. Immunol.* 124 (2009) 573–582 (e1–9).
- [27] R. Halwani, A. Vazquez-Tello, Y. Sumi, M.A. Pureza, A. Bahammam, H. Al-Jahdali, et al., Eosinophils induce airway smooth muscle cell proliferation, *J. Clin. Immunol.* 33 (2013) 595–604.
- [28] B. Bosnjak, B. Stelzmueller, K.J. Erb, M.M. Epstein, Treatment of allergic asthma: modulation of Th2 cells and their responses, *Respir. Res.* 12 (2011) 114.
- [29] G.G. Brusselle, J.C. Kips, J.H. Tavernier, J.G. van der Heyden, C.A. Cuvelier, R.A. Pauwels, et al., Attenuation of allergic airway inflammation in IL-4 deficient mice, *Clin. Exp. Allergy* 24 (1994) 73–80.
- [30] D. Vercelli, H.H. Jabara, B.W. Lee, N. Woodland, R.S. Geha, D.Y. Leung, Human recombinant interleukin 4 induces Fc epsilon R2/CD23 on normal human monocytes, *J. Exp. Med.* 167 (1988) 1406–1416.
- [31] K.F. Chung, P.J. Barnes, Cytokines in asthma, *Thorax* 54 (1999) 825–857.
- [32] M. Wills-Karp, J. Luyimbazi, X. Xu, B. Schofield, T.Y. Neben, C.L. Karp, et al., Interleukin-13: central mediator of allergic asthma, *Science* 282 (1998) 2258–2261.
- [33] Z. Zhu, R.J. Homer, Z. Wang, Q. Chen, G.P. Geba, J. Wang, et al., Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production, *J. Clin. Invest.* 103 (1999) 779–788.
- [34] A.K. Behera, M. Kumar, R.F. Lockey, S.S. Mohapatra, Adenovirus-mediated interferon gamma gene therapy for allergic asthma: involvement of interleukin 12 and STAT4 signaling, *Hum. Gene Ther.* 13 (2002) 1697–1709.
- [35] J.J. Lee, D. Dimina, M.P. Macias, S.I. Ochkur, M.P. McGarry, K.R. O'Neill, et al., Defining a link with asthma in mice congenitally deficient in eosinophils, *Science* 305 (2004) 1773–1776.
- [36] B.L. Bradley, M. Azzawi, M. Jacobson, B. Assoufi, J.V. Collins, A.M. Irani, et al., Eosinophils, T-lymphocytes, mast cells, neutrophils, and macrophages in bronchial biopsy specimens from atopic subjects with asthma: comparison with biopsy specimens from atopic subjects without asthma and normal control subjects and relationship to bronchial hyperresponsiveness, *J. Allergy Clin. Immunol.* 88 (1991) 661–674.
- [37] M. Lommatzsch, Airway hyperresponsiveness: new insights into the pathogenesis, *Semin. Respir. Crit. Care Med.* 33 (2012) 579–587.
- [38] D.F. Rogers, Physiology of airway mucus secretion and pathophysiology of hypersecretion, *Respir. Care* 52 (2007) 1134–1146 (discussion 46–9).
- [39] C.W. Davis, B.F. Dickey, Regulated airway goblet cell mucin secretion, *Annu. Rev. Physiol.* 70 (2008) 487–512.
- [40] C.L. Ordóñez, R. Khashayar, H.H. Wong, R. Ferrando, R. Wu, D.M. Hyde, et al., Mild and moderate asthma is associated with airway goblet cell hyperplasia and abnormalities in mucin gene expression, *Am. J. Respir. Crit. Care Med.* 163 (2001) 517–523.
- [41] A. Ozier, B. Allard, I. Bara, P.O. Girodet, T. Trian, R. Marthan, et al., The pivotal role of airway smooth muscle in asthma pathophysiology, *J. Allergy* 2011 (2011) 742710.
- [42] M. Kudo, Y. Ishigatsubo, I. Aoki, Pathology of asthma, *Front. Microbiol.* 4 (2013) 263.
- [43] P.G. Woodruff, G.M. Dolganov, R.E. Ferrando, S. Donnelly, S.R. Hays, O.D. Solberg, et al., Hyperplasia of smooth muscle in mild to moderate asthma without changes in cell size or gene expression, *Am. J. Respir. Crit. Care Med.* 169 (2004) 1001–1006.
- [44] G.N. Xu, K. Yang, Z.P. Xu, L. Zhu, L.N. Hou, H. Qi, et al., Protective effects of anisodamine on cigarette smoke extract-induced airway smooth muscle cell proliferation and tracheal contractility, *Toxicol. Appl. Pharmacol.* 262 (2012) 70–79.
- [45] R.A. Panettieri Jr., Cellular and molecular mechanisms regulating airway smooth muscle proliferation and cell adhesion molecule expression, *Am. J. Respir. Crit. Care Med.* 158 (1998) S133–S140.
- [46] J.K. Burgess, J.H. Lee, Q. Ge, E.E. Ramsay, M.H. Poniris, J. Parmentier, et al., Dual ERK and phosphatidylinositol 3-kinase pathways control airway smooth muscle proliferation: differences in asthma, *J. Cell. Physiol.* 216 (2008) 673–679.
- [47] W. Gao, L. Cai, X. Xu, J. Fan, X. Xue, X. Yan, et al., Anti-CTGF single-chain variable fragment dimers inhibit human airway smooth muscle (ASM) cell proliferation by down-regulating p-Akt and p-mTOR levels, *PLoS One* 9 (2014), e113980.
- [48] N.S. Redhu, L. Shan, D. Al-Subait, H.L. Ashdown, H. Movassagh, B. Lamkhoui, et al., IgE induces proliferation in human airway smooth muscle cells: role of MAPK and STAT3 pathways, *Allergy Asthma Clin. Immunol.* 9 (2013) 41.
- [49] R. Halwani, J. Al-Abri, M. Beland, H. Al-Jahdali, A.J. Halayko, T.H. Lee, et al., CC and CXC chemokines induce airway smooth muscle proliferation and survival, *J. Immunol.* 186 (2011) 4156–4163.
- [50] A. Whelchel, J. Evans, J. Posada, Inhibition of ERK activation attenuates endothelin-stimulated airway smooth muscle cell proliferation, *Am. J. Respir. Cell Mol. Biol.* 16 (1997) 589–596.
- [51] K.J. Woo, Y.J. Jeong, J.W. Park, T.K. Kwon, Chrysin-induced apoptosis is mediated through caspase activation and Akt inactivation in U937 leukemia cells, *Biochem. Biophys. Res. Commun.* 325 (2004) 1215–1222.
- [52] E. Pichichero, R. Cicconi, M. Mattei, A. Canini, Chrysin-induced apoptosis is mediated through p38 and Bax activation in B16-F1 and A375 melanoma cells, *Int. J. Oncol.* 38 (2011) 473–483.