

# $\beta$ -sitosterol targets glucocorticoid receptor to reduce airway inflammation and remodeling in allergic asthma

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## ABSTRACT

**Introduction:** In most asthma patients, symptoms are controlled by treatment with glucocorticoid, but long-term or high-dose use can produce adverse effects. Therefore, it is crucial to find new therapeutic strategies.  $\beta$ -sitosterol could suppress type II inflammation in ovalbumin (OVA)-induced mice, but its mechanisms have remained unclear.

**Methods:** A binding activity of  $\beta$ -sitosterol with glucocorticoid receptor (GR) was analyzed by molecular docking. Human bronchial epithelial cells (BEAS-2B) and human bronchial smooth muscle cells (HBSMC) were treated with different concentrations (0, 1, 5, 10, 20, and 50  $\mu$ g/mL) of  $\beta$ -sitosterol for suitable concentration selection. In transforming growth factor (TGF)- $\beta$ 1 treated BEAS-2B and HBSMC, cells were treated with 20  $\mu$ g/mL  $\beta$ -sitosterol or dexamethasone (Dex) to analyze its possible mechanism. In OVA-induced mice, 2.5 mg/kg  $\beta$ -sitosterol or Dex administration was performed to analyze the therapeutic mechanism of  $\beta$ -sitosterol. A GR antagonist RU486 was used to confirm the mechanism of  $\beta$ -sitosterol in the treatment of asthma.

**Results:** A good binding of  $\beta$ -sitosterol to GR (score =  $-8.2$  kcal/mol) was found, and the GR expression was upregulated with  $\beta$ -sitosterol dose increase in BEAS-2B and HBSMC. Interleukin (IL)-25 and IL-33 secretion was significantly decreased by  $\beta$ -sitosterol in the TGF- $\beta$ 1-induced BEAS-2B, and the levels of collagen 1A and  $\alpha$ -smooth muscle actin (SMA) were reduced in the TGF- $\beta$ 1-induced HBSMC. In the OVA-challenged mice,  $\beta$ -sitosterol treatment improved airway inflammation and remodeling through suppressing type II immune response and collagen deposition. The therapeutic effects of  $\beta$ -sitosterol were similar to Dex treatment *in vitro* and *in vivo*. RU486 treatment clearly hampered the therapeutic effects of  $\beta$ -sitosterol in the TGF- $\beta$ 1-induced cells and OVA-induced mice.

**Conclusion:** This study identified that  $\beta$ -sitosterol binds GR to perform its functions in asthma treatment.  $\beta$ -sitosterol represent a potential therapeutic drug for allergic asthma.

## 1. Introduction

Allergic asthma is the most common asthma characterized by airway inflammation and airway hyperresponsiveness (AHR), accounting for 80% of childhood asthma [1]. Allergic asthma is mediated by type II immune response, in which helper type 2 (Th2) cells and their cytokines, interleukin (IL)-4, IL-5 and IL-13, play a central role [2]. Upon exposure to an allergen, airway epithelial cells secrete IL-25 and IL-33 to activate natural immune cells, such as dendritic cells, and further initiate type II immune response. The secretion of type II cytokines induces the aggregation of eosinophils, neutrophils, lymphocytes, and macrophages

and in turn, affects airway remodeling [2–4]. Airway remodeling is characterized by matrix deposition and enhanced smooth muscle mass in the airways. Additionally, collagen deposition and goblet cell hyperplasia contribute to airway remodeling in asthma [5].

In most asthma patients, symptoms are well controlled by glucocorticoid treatment (e.g., dexamethasone and prednisolone), but long-term or high-dose use can produce adverse drug reactions (ADRs), such as mental disorders, body deformation, hypertension, diabetes, osteoporosis, etc. [2,6,7]. As the mainstay of asthma treatment, glucocorticoids have significant anti-inflammatory, immunosuppressive and immunomodulatory effects. The biological action of glucocorticoids is

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mediated by glucocorticoid receptor (GR), which GR is a ligand-activated transcription factor belonging to the steroid receptor superfamily [8]. In clinical treatment, GR is one of the targets for many approved drugs [9]. However, the existence of ADRs and the inability of asthmatics patients to control symptoms generate the need for new therapeutic strategies.

Chemically, the structure of phytosterols is similar to that of steroids. Beta( $\beta$ )-sitosterol (24-ethyl-5-cholestene-3-ol), one of the phytosterols, is found in many plants, and has shown to possess antioxidant, anti-inflammatory, antitumor, and anti-asthmatic activities [7,10–13]. In a model of ovalbumin (OVA)-induced asthma,  $\beta$ -sitosterol inhibited Th2-driven airway inflammation [10,13], but its mechanisms of anti-inflammatory effects have not been explained. Based on the chemical structure association between dexamethasone (Dex) and  $\beta$ -sitosterol, Leng et al. have found that  $\beta$ -sitosterol could bind the ligand binding domain of GR by molecular docking and inhibit cell viability of Hela and HepG2 cells [7]. However, it is unclear whether  $\beta$ -sitosterol targets GR to improve airway inflammation and remodeling in allergic asthma treatment.

In this study, the effects of  $\beta$ -sitosterol on GR expression in human normal bronchial epithelial cells and human bronchial smooth muscle cells (HBSMC) were analyzed. Furthermore, the effects of  $\beta$ -sitosterol on airway inflammation and remodeling by regulating GR were analyzed in transforming growth factor (TGF)- $\beta$ 1-treated cells and OVA-induced mice. The effects of GR antagonist RU486 combined with  $\beta$ -sitosterol were investigated to confirm the mechanism of  $\beta$ -sitosterol *in vitro* and *in vivo*. This study might provide a new drug for the treatment of allergic asthma.

## 2. Materials and methods

### 2.1. Molecular docking

The three-dimensional (3D) structure of GR was downloaded from the PDB database (<https://www.rcsb.org/>) and saved as in a PDB format. The 3D structures of  $\beta$ -sitosterol (CAS:83-46-5) and Dex (CAS: 50-02-2) were downloaded from the PubChem (<https://pubchem.ncbi.nlm.nih.gov/>) and saved in the PDB format. According to a reported study [14], mechanical optimization, hydrogenation and charging of the ligand were performed by the UCSF chimera software (<https://www.cgl.ucsf.edu/chimera/>). The AutoDock Vina tool was performed to obtain molecular docking. A grid box was produced, being large enough to cover the entire protein binding site and allow all ligands to move freely. The docking score indicated the intermolecular energy (kcal/mol), representing the stability between the ligand and receptor.

### 2.2. Cell culture

Human bronchial epithelial cells (BEAS-2B) and HBSMC were purchased from Procell (Wuhan, China). They were cultured in a Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin. The cells were cultivated on plastic plates and cultured at 37 °C and 5% CO<sub>2</sub>, and the medium was changed every three days. For all experiments, the same cell line was used to avoid changes in cell activity and viability that could result from repeated thawing and passage. Plates with 50–60% confluence were used for the following experiments.

### 2.3. Toxicity of $\beta$ -sitosterol for cells

Different concentrations (0, 1, 5, 10, 20, and 50  $\mu$ g/mL) of  $\beta$ -sitosterol ( $\beta$ -S1270, Sigma-Aldrich) were solved in 0.1% dimethyl sulfoxide (DMSO). The BEAS-2B cells and HBSMC cells ( $1 \times 10^7$  cells/well) were seeded in 96-well plates and treated with different doses of  $\beta$ -sitosterol for 24 h.

Cell viability was measured using a CCK8 kit. According to the kit's

instruction, 10  $\mu$ L CCK8 solution was added to the cells, which were cultured for 4 h. Then, the absorbance was recorded at 450 nm using a microplate reader (ELx808, BioTek).

### 2.4. Effects of $\beta$ -sitosterol on normal cells

The BEAS-2B and HBSMC cells ( $1 \times 10^7$  cells/well) were treated with 20  $\mu$ g/mL  $\beta$ -sitosterol for 24 h to analyze the effects of  $\beta$ -sitosterol on normal cells. The cell viability and GR expression in cells were observed.

### 2.5. Cell groups and treatment

For BEAS-2B and HBSMC cells, they were respectively divided into five groups, including the control group, dexamethasone (Dex) group,  $\beta$ -sitosterol group, RU486 plus  $\beta$ -sitosterol group, and vehicle group.

In the control group, the cells were cultured in normal conditions for 48 h.

In the Dex and  $\beta$ -sitosterol groups, the cells were cultured in a medium with 10 ng/mL recombinant human TGF- $\beta$ 1 ( $\beta$ -91701ES08, YEASEN, China) for 24 h, which was then changed to a fresh medium with 20  $\mu$ g/mL Dex [9] or 20  $\mu$ g/mL  $\beta$ -sitosterol for 24 h.

In the RU486 plus  $\beta$ -sitosterol group, the cells were cultured in a medium with 10 ng/mL TGF- $\beta$ 1 for 24 h, which was then changed to a fresh medium with 30 nM RU486 [15] and 20  $\mu$ g/mL  $\beta$ -sitosterol for 24 h. RU486 is a GR antagonist with high affinity for both GR and progesterone receptor (PR) and a strong *in vitro* and *in vivo* GR antagonist activity [16].

In the vehicle group, the cells were cultured in a medium with 10 ng/mL TGF- $\beta$ 1 for 24 h, which was then changed to a fresh medium with 0.1% DMSO for 24 h.

### 2.6. Animal and asthma model

Fifty-six C57BL/6 J male mice (Jinan Pengyue experimental animal breeding Co., Ltd), 6–8-weeks-old, were used in this study. All mice were housed under specific pathogen-free conditions.

After one week of adaptation, the asthma model was created as in a reported description [16,17]. In brief, mice were sensitized intraperitoneally (i.p.) with 20  $\mu$ g OVA ( $\beta$ -MFCD00889188, Macklin, dissolved in PBS) and 1 mg Aluminium hydroxide in 200  $\mu$ L saline on days 1 and days 8, and then challenged by the inhalation of 5% (V/V) OVA for 30 min on days 15, 16, and 17 after the initial sensitization with OVA. For the OVA inhalation, mice were administrated using an ultrasonic nebulizer (NE-U17, Omron) in a whole-body exposure chamber. Twenty-four hours after the last challenge, the mice were performed different experiments.

### 2.7. Effects of $\beta$ -sitosterol on normal mice

Sixteen C57BL/6 J male mice (8 mice in each group) received 0.1% DMSO or 2.5 mg/kg  $\beta$ -sitosterol every day for 10 days by intranasal injection to analyze the effects of  $\beta$ -sitosterol on normal mice. Lung pathology was observed by hematoxylin and eosin (HE) staining, and GR expression in lung tissues was observed by immunofluorescence staining.

### 2.8. Animal treatment and groups

All mice were randomly divided into the following groups (8 mice in each group): control group, Dex group,  $\beta$ -sitosterol group, RU486 plus  $\beta$ -sitosterol group, and vehicle group.

In the control group, mice received with 20  $\mu$ L PBS in 200  $\mu$ L saline on days 1 and 8 intraperitoneally, and then were nebulized with 5% PBS for 30 min on days 15, 16 and 17 days after the sensitization.

In the Dex group and  $\beta$ -sitosterol group, mice were sensitized and challenged with OVA and intranasally treated with 2.5 mg/kg Dex or

2.5 mg/kg  $\beta$ -sitosterol solution every day [10] from days 8–17 prior to the challenge with OVA.

In the RU486 plus  $\beta$ -sitosterol group, mice were sensitized and challenged with OVA and treated orally with 20 mg/kg RU486 [18], and intranasally with 2.5 mg/kg  $\beta$ -sitosterol every day from days 8–17 prior to the challenge with OVA.

In the vehicle group, mice were sensitized and challenged with OVA, and intranasally treated with 1% DMSO solution every day from days 8–17 prior to the challenge with OVA.

## 2.9. AHR

According to a previous description [19], the AHR of mice was assessed at 24 h after the last challenge with OVA. Briefly, the mice were anesthetized with pentobarbital sodium (45 mg/kg), and a tracheostomy tube was placed in the trachea of the mice. A Sci-Req FlexiVent machine was used to mechanically ventilate under 150 breaths/min and a tidal volume of 2 mL. Airway resistance was determined at baseline and after the administration of increasing doses (0, 12.5, 25, 50, and 100 mg/mL) of nebulized methacholine (CAS: 62-51-1, Macklin).

## 2.10. Bronchioalveolar lavage (BAL) and enzyme-linked immunosorbent assay (ELISA)

After assessing the AHR, bronchioalveolar lavage fluids (BALFs) were obtained through the injection of 1 mL PBS into the mice's lung via the tracheal tube. A total number of cells in the BALFs was counted by trypan blue dye exclusion, and the counts of eosinophils, neutrophils and lymphocytes were analyzed using Wright stain solution (#G1040, Solarbio).

After treatment, the BEAS-2B cells were collected to analyze the levels of IL-25 (#ab272200, Abcam) and IL-33 (#PI631, Beyotime). After centrifugation ( $\times 800$  g, 15 min) of BALFs, supernatants were collected to analyze the levels of IL-4 (#PI612, Beyotime), IL-5 (#PI620, Beyotime), and IL-13 (#PI539, Beyotime). The cytokine levels were analyzed by ELISA kits.

## 2.11. HE and Masson staining

After the collection of BALFs, all mice were sacrificed and lung tissues were injected with a small amount of 4% paraformaldehyde, which were then fixed in 4% paraformaldehyde for 48 h. The fixed tissues were embedded in paraffin and cut into serial sections (3  $\mu$ m). According to the modified hematoxylin and eosin (HE) staining method [20], the sections were stained with hematoxylin and eosin (Solarbio, China). According to Masson trichrome staining method [20], the sections were stained with Masson solution (Solarbio, China). The results were observed under a light microscope.

## 2.12. Immunofluorescence staining

According to the reported protocols [21,22], cells of each group were trypsinized and seeded onto 10-cm culture dishes, each containing 15 to 20 sterilized glass coverslips. 20  $\mu$ L of suspended cells were applied to each coverslip for 10 min and then fixed with 4% paraformaldehyde for 10 min. According to the protocols, the cells and lung sections were washed and blocked with BS-T containing 1% BSA for 30 min, and incubated with GR antibody (ab183127, 1:500, Abcam) for overnight at 4 °C. After washing with TBS, the cells and sections were incubated with Cy3-conjugated goat anti-rabbit IgG (H + L) antibody (ab6939, 1:2000, Abcam) for 60 min. Nuclei of cells and tissues were stained with 4', 6-diamidino-2-phenylindole (DAPI) for 60s. After washing and sealing, the results were obtained by a confocal microscope.

## 2.13. Western blot

As referenced in reported protocols [23], proteins from frozen cells and lung tissues were collected using RIPA buffer and separated with 12% SDS-PAGE. The separated proteins were transferred to polyvinylidene fluoride (PVDF) membranes, and incubated with appropriate primary antibodies for overnight at 4 °C. The antibodies were as follows: GR (#ab183127, 1:1000, Abcam), collagen 1A (#72026, 1:1000, Cell Signaling Technology),  $\alpha$ -smooth muscle actin (SMA) (#50513, 1:1000, Cell Signaling Technology) and TGF- $\beta$ 1 (#ab215725, 1:1000, Abcam), GAPDH (#8884, 1:1000, Cell Signaling Technology). The second antibody HRP-linked anti-rabbit IgG (1:1000, Cell Signaling Technology) was cultured with the washed membranes for 60 min at 37 °C. The signals were visualized using an enhanced chemiluminescence reagent kit.

## 2.14. Statistical analysis

Data were analyzed by GraphPad Prism 6.0 software (GraphPad Software Inc., CA), and showed as mean  $\pm$  standard deviation. Significance testing was performed by one-way analysis of variance following the Tukey test among groups. Statistical significance was considered as a value of  $p < 0.05$ .

## 3. Results

### 3.1. $\beta$ -sitosterol promoted GR expression in BEAS-2B and HBSMC

As shown in Fig. 1A, a good binding activity of Dex (score =  $-11.2$  kcal/mol) with GR and  $\beta$ -sitosterol (score =  $-8.2$  kcal/mol) with GR was separately found through molecular docking. The more negative the value, the more stable the binding. Different concentrations (0, 1, 5, 10, 20 and 50  $\mu$ g/mL) of  $\beta$ -sitosterol were used for 24 h in the BEAS-2B and HBSMC to choose the appropriate dose of  $\beta$ -sitosterol. By observing the cell viability (Fig. 1B), we found that 50  $\mu$ g/mL  $\beta$ -sitosterol significantly suppressed cell viability when compared with other doses ( $p < 0.01$ ). Additionally, the expression levels of GR in the 10 ng/mL TGF- $\beta$ 1-treated BEAS-2B and HBSMC were upregulated with  $\beta$ -sitosterol dose treatment (Fig. 1C). We selected 20  $\mu$ g/mL  $\beta$ -sitosterol in the following experiment to further study the mechanism of  $\beta$ -sitosterol in treatment of allergic asthma.

### 3.2. Effects of $\beta$ -sitosterol on normal cells and mice

After the treatment with 0.1% DMSO or 20  $\mu$ g/mL  $\beta$ -sitosterol for 24 h, the cell viability of BEAS-2B and HBSMC was measured (Fig. 2A). No significant difference was found among the groups, suggesting that 20  $\mu$ g/mL  $\beta$ -sitosterol was feasible. Cellular GR expression was measured by western blot (Fig. 2B). We found that its expression was significantly increased after  $\beta$ -sitosterol treatment compared with normal control cells ( $p < 0.05$ ). Similarly, the lung pathology of mice was observed after 2.5 mg/kg  $\beta$ -sitosterol or 0.1% DMSO treatment for 10 days by HE staining (Fig. 2C), and no inflammatory infiltration was found. In mice, the GR expressions were measured by immunofluorescence (Fig. 2D), and their expressions were clearly increased after  $\beta$ -sitosterol treatment by contrast to the normal mice ( $p < 0.05$ ).

### 3.3. $\beta$ -sitosterol suppressed inflammation through GR in TGF- $\beta$ 1-induced BEAS-2B

In different treated BEAS-2B cells, the expression levels of GR were measured through immunofluorescence (Fig. 3A) and western blot (Fig. 3B). After stimulated with 10 ng/mL TGF- $\beta$ 1, the GR levels significantly downregulated in the BEAS-2B cells ( $p < 0.05$ ). When contrasted to the vehicle-treated cells, the GR levels were significantly increased after administration with 20  $\mu$ g/mL Dex or  $\beta$ -sitosterol ( $p <$

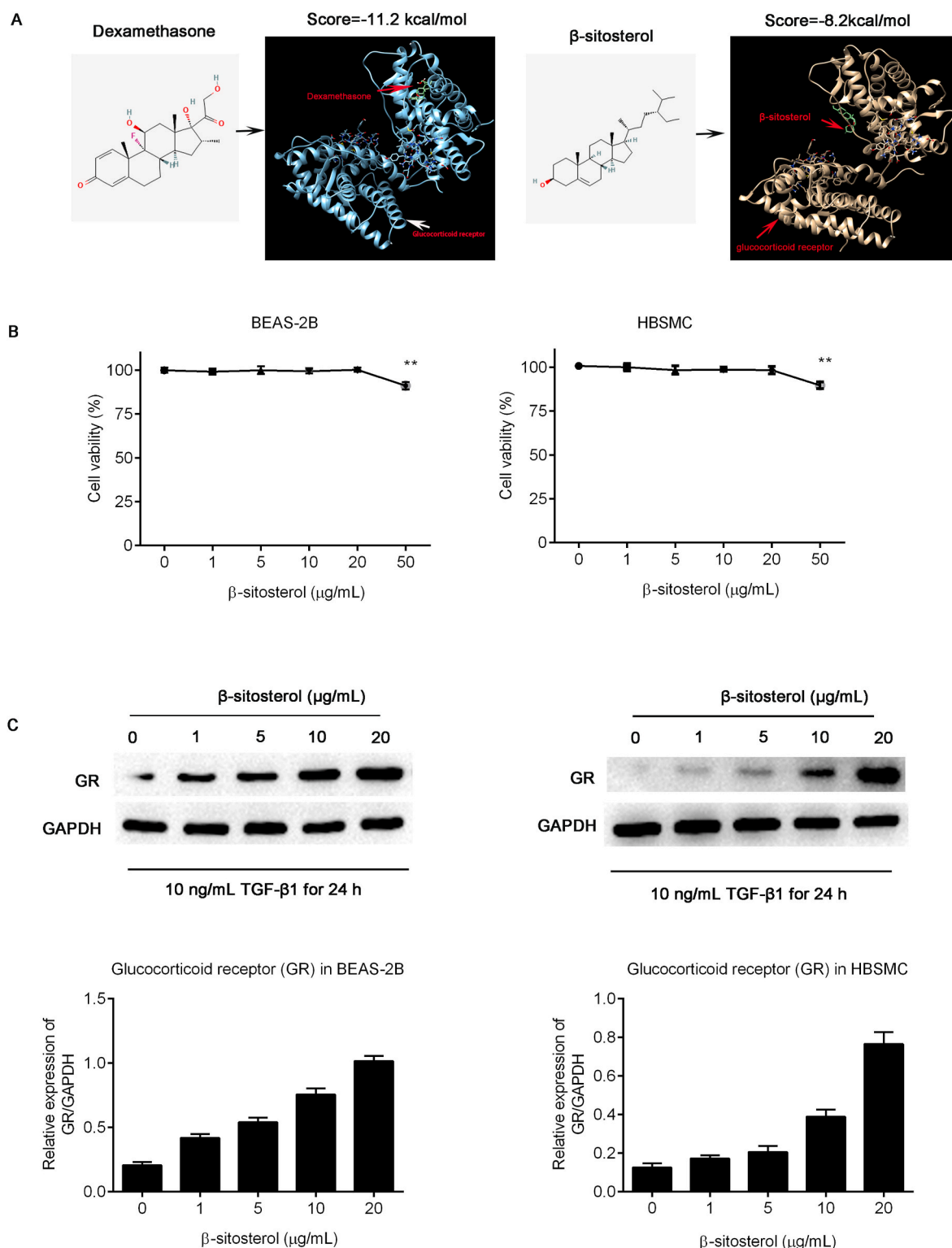


**Fig. 1.** Glucocorticoid receptor (GR) expression level increased with  $\beta$ -sitosterol dose increase in BEAS-2B and HBSMC cells. (A) The binding of dexamethasone (Dex) or  $\beta$ -sitosterol to GR was analyzed by molecular docking, and good activity of  $\beta$ -sitosterol regarding GR was shown (score = -8.2 kcal/mol); (B) The BEAS-2B and HBSMC were treated with different concentrations of  $\beta$ -sitosterol for 24 h, and the cell viability was analyzed using a CCK-8 kit, and 50  $\mu$ g/mL  $\beta$ -sitosterol significantly downregulated the cells viability; (C) Different concentrations of  $\beta$ -sitosterol were used to analyze changes of GR in 10 ng/mL TGF- $\beta$ 1-treated BEAS-2B and HBSMC. The GR expression levels were measured by western blot. Three parallel experiments were performed. \*\* $p < 0.01$ , vs. control cells.

0.01). The application of RU486 (30 nM) clearly suppressed the roles of  $\beta$ -sitosterol (20  $\mu$ g/mL) on GR expression ( $p < 0.01$ ). The secretions of IL-25 and IL-33 in the BEAS-2B cells were shown in Fig. 3C. The contents of IL-25 and IL-33 were markedly increased after the treatment

with TGF- $\beta$ 1 compared with the control cells ( $p < 0.01$ ). After the treatment with Dex or  $\beta$ -sitosterol, their contents were clearly decreased ( $p < 0.01$ ). However, RU486 treatment hampered the effects of  $\beta$ -sitosterol on IL-25 and IL-33 levels ( $p < 0.01$ ).

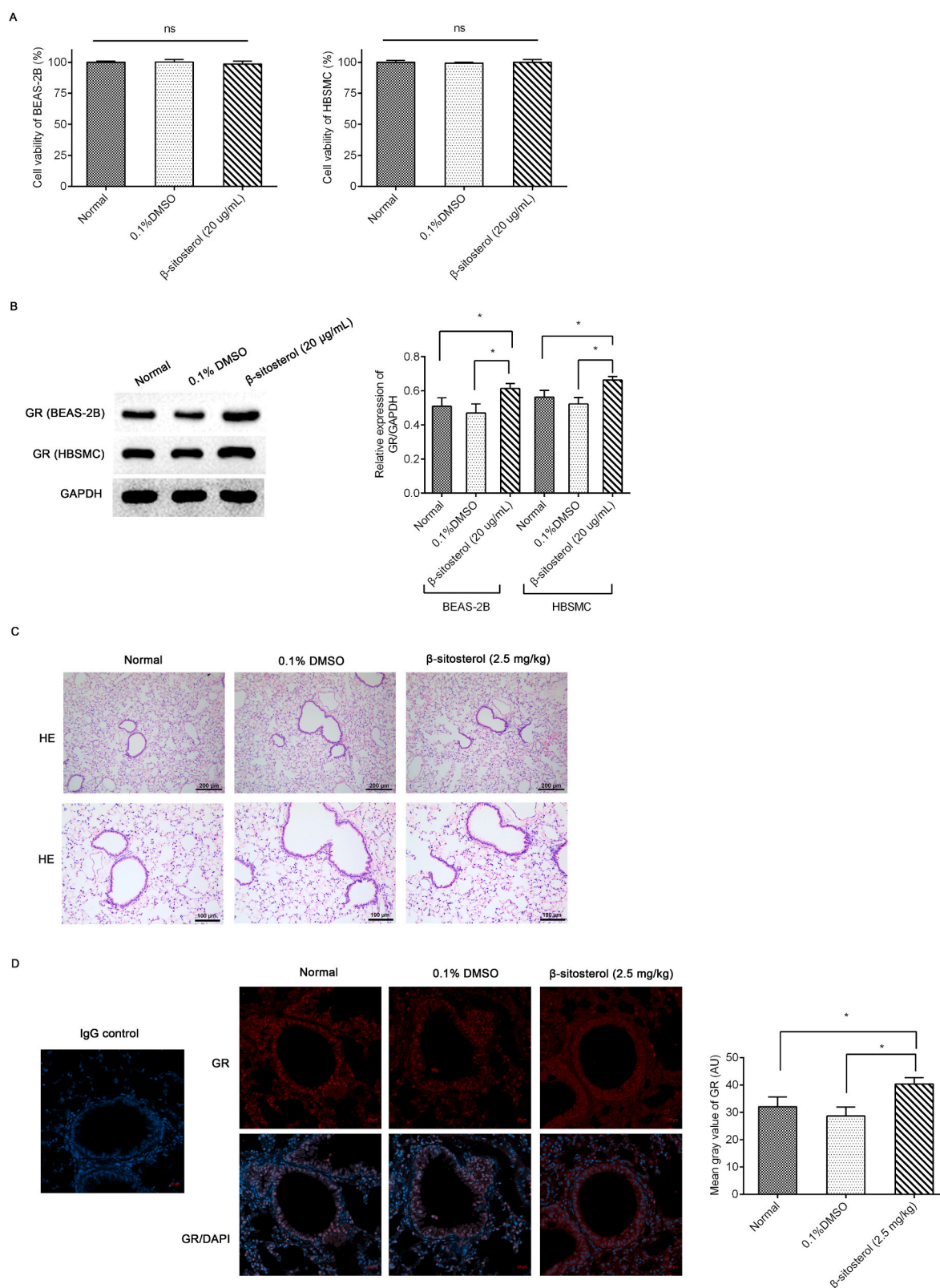


**Fig. 2.**  $\beta$ -sitosterol increased GR expression in normal cells and mice. The BEAS-2B and HBSMC cells were treated with 0.1% DMSO or 20  $\mu$ g/mL  $\beta$ -sitosterol for 24 h. The male mice were treated with 0.1% DMSO or 2.5 mg/kg/d  $\beta$ -sitosterol for 10 days. (A) Cell viability of BEAS-2B and HBSMC was measured by a CCK-8 kit; (B) The GR expression in cells was measured by immunofluorescence; (C) The lung pathology was observed by HE; (D) GR expressions in lung tissues were observed by immunofluorescence. Three parallel experiments were performed. ns: no significance, \* $p < 0.05$ .

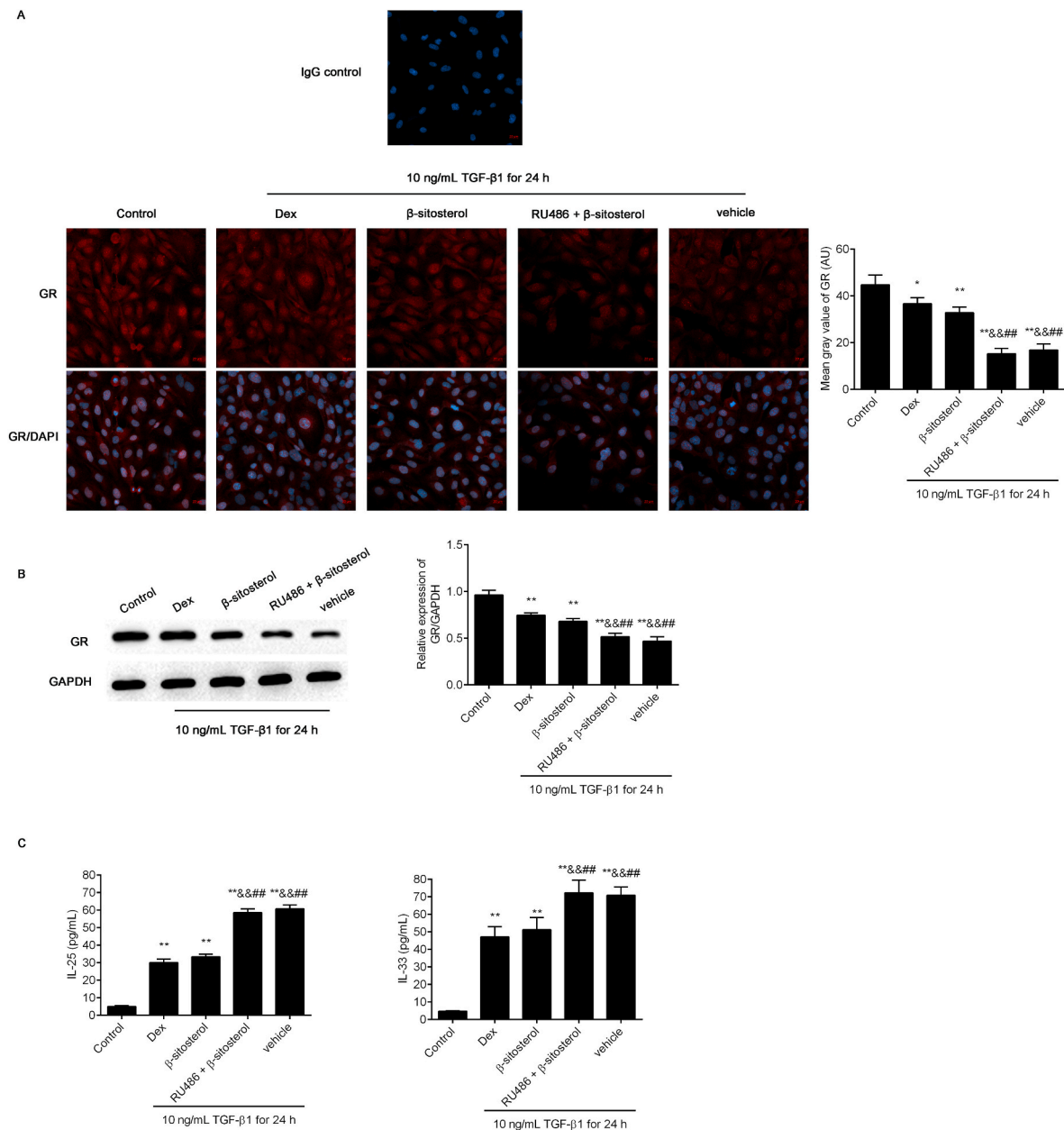


**Fig. 3.**  $\beta$ -sitosterol suppressed inflammation through GR in TGF- $\beta$ 1-induced BEAS-2B cells. The GR expression in BEAS-2B cells was measured by immunofluorescence (A) and western blot (B). (C) The levels of IL-25 and IL-33 in cells were analyzed by ELISA. 10 ng/mL TGF- $\beta$ 1 was used to stimulate cells for 24 h, which were then treated with 20  $\mu$ g/mL Dex or 20  $\mu$ g/mL  $\beta$ -sitosterol for 24 h. The Dex or  $\beta$ -sitosterol was dissolved in 0.1% DMSO. A GR antagonist RU486 (30 nM) was used to confirm the mechanism of  $\beta$ -sitosterol in BEAS-2B cells. Three parallel experiments were performed. vs. control group, \*\* $p$  < 0.01; vs. Dex group, &# $p$  < 0.01; vs.  $\beta$ -sitosterol group, ## $p$  < 0.01.

### 3.4. $\beta$ -sitosterol suppressed fibrosis-related proteins through GR in TGF- $\beta$ 1-induced HBSMC

In different treated HBSMC, a similar trend was found in the expression levels of GR (Fig. 4A and B). In addition, the changes in collagen 1A and  $\alpha$ -SMA protein expression were compared in the HBSMC (Fig. 4C). The expression levels of collagen 1A and  $\alpha$ -SMA were markedly increased after the treatment with TGF- $\beta$ 1 compared with the control cells ( $p$  < 0.01). After the treatment with Dex or  $\beta$ -sitosterol, their expression levels were clearly decreased compared with the vehicle group ( $p$  < 0.01). RU486 treatment hampered the effects of  $\beta$ -sitosterol on collagen 1A and  $\alpha$ -SMA expression ( $p$  < 0.05).

### 3.5. $\beta$ -sitosterol inhibited Th2 inflammation in OVA-induced mice

In order to further study the mechanism of  $\beta$ -sitosterol in allergic asthma, the allergic asthma mice model was induced by OVA, and intranasally treated with 2.5 mg/kg Dex or 2.5 mg/kg  $\beta$ -sitosterol solution every day from days 8–17 prior to the challenge with OVA (Fig. 5A). Airway resistance was measured in different groups (Fig. 5B). After the OVA challenge, the airway resistance was significantly upregulated contrasted to the control mice. When mice were treated with 2.5 mg/kg Dex or  $\beta$ -sitosterol, the airway resistance of mice was clearly downregulated ( $p$  < 0.01). No significant difference was found between the Dex and  $\beta$ -sitosterol groups. However, the airway resistance of mice in the RU486 +  $\beta$ -sitosterol group was significantly higher than that in the  $\beta$ -sitosterol group ( $p$  < 0.01).

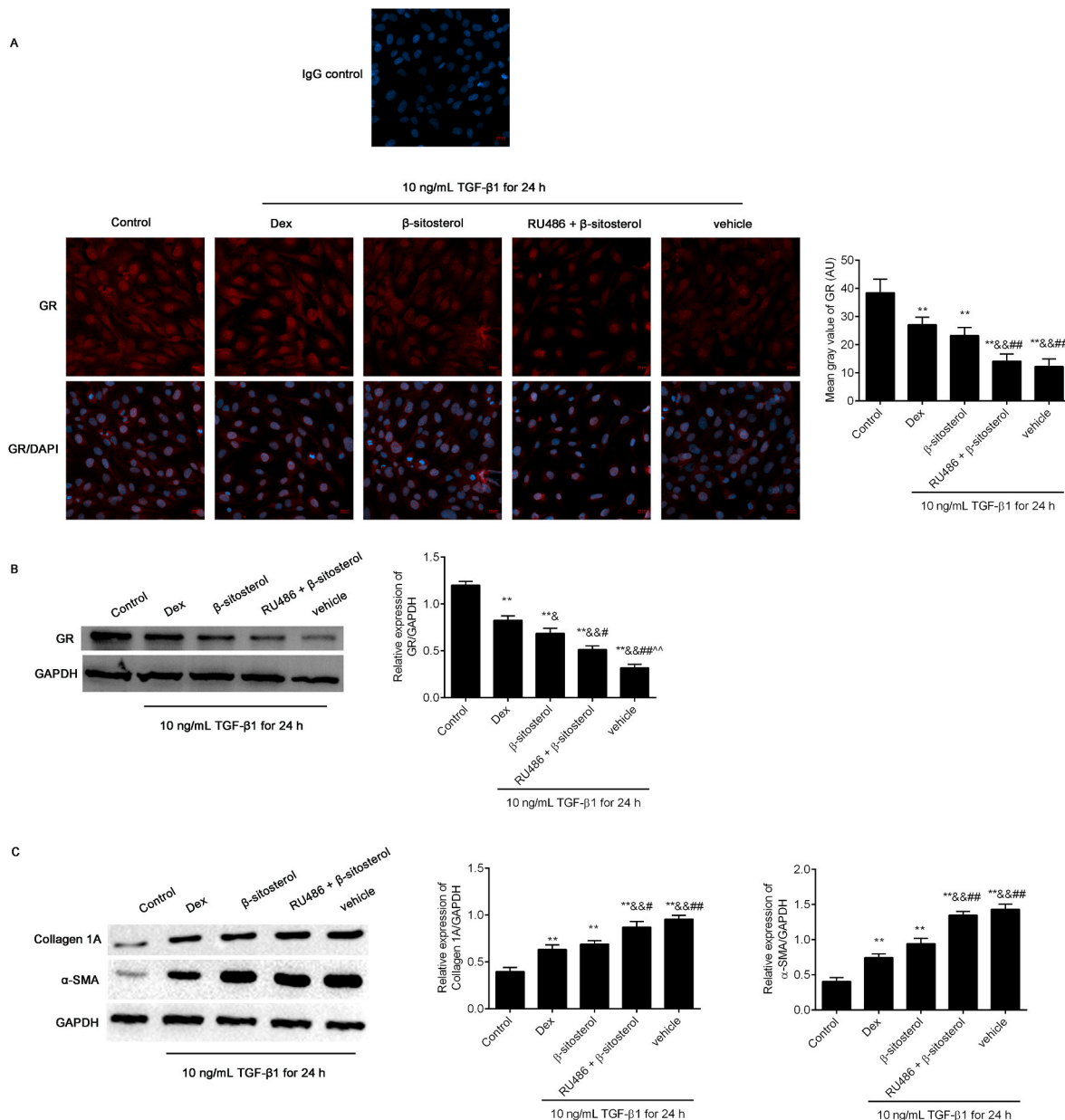


**Fig. 4.**  $\beta$ -sitosterol suppressed fibrosis-related proteins through GR in TGF- $\beta$ 1-induced HBSMC. The GR expression in HBSMC was measured by immunofluorescence (A) and western blot (B). (C) The expression levels of collagen 1A and  $\alpha$ -SMA in cells were analyzed by western blot. 10 ng/mL TGF- $\beta$ 1 was used to stimulate cells for 24 h, then treated with 20  $\mu$ g/mL Dex or 20  $\mu$ g/mL  $\beta$ -sitosterol for 24 h. The Dex or  $\beta$ -sitosterol was dissolved in 0.1% DMSO. A GR antagonist RU486 (30 nM) was used to confirm the mechanism of  $\beta$ -sitosterol in HBSMC. Three parallel experiments were performed. vs. control group, \*\* $p < 0.01$ ; vs. Dex group,  $^{\&}$  $p < 0.05$ ,  $^{\&\&}$  $p < 0.01$ ; vs.  $\beta$ -sitosterol group,  $^{\#}$  $p < 0.05$ ,  $^{\#\#}$  $p < 0.01$ ; vs. RU486+ $\beta$ -sitosterol group,  $p < 0.05$ .

The numbers of eosinophils, lymphocytes and neutrophils in BALFs were counted (Fig. 5C), and we found their numbers were clearly increased in OVA-induced mice ( $p < 0.01$ ). In the Dex or  $\beta$ -sitosterol group, the numbers of eosinophils, lymphocytes and neutrophils were notably decreased compared with the vehicle group ( $p < 0.01$ ). Compared with the Dex group, the numbers of eosinophils and lymphocytes were upregulated in the  $\beta$ -sitosterol group ( $p < 0.05$ ). No significant difference was found in the numbers of neutrophils and levels of IL-4, IL-5 and IL-13 between the Dex and  $\beta$ -sitosterol groups. In the same way, the RU486 (20 mg/kg) oral treatment prevented the effects of  $\beta$ -sitosterol ( $p < 0.01$ ). The levels of IL-4, IL-5 and IL-13 in BALFs were also measured in different groups (Fig. 5D). A similar trend was shown in these inflammatory cytokines in that Dex or  $\beta$ -sitosterol treatment clearly suppressed the secretion of these inflammatory cytokines, as induced by OVA. The application of RU486 blocked the roles of

$\beta$ -sitosterol on these inflammatory cytokines ( $p < 0.01$ ).

### 3.6. $\beta$ -sitosterol improved airway inflammation and fibrosis in lung tissue of OVA-induced mice

Pathological changes of lung tissue were observed through HE (Fig. 6A) and Masson (Fig. 6B). As shown in Fig. 6A, the OVA challenge reduced inflammatory infiltration (black arrow) in the lung tissue of mice, but the inflammation response was weakened in the Dex or  $\beta$ -sitosterol group. However, the administration of RU486 aggravated the inflammatory infiltration in the RU486 +  $\beta$ -sitosterol group. In Fig. 6B, collagen fibers (black arrow) were significantly increased in lung tissues after OVA stimulation ( $p < 0.01$ ). Compared with the vehicle treatment, the collagen fibers clearly decreased after the Dex or  $\beta$ -sitosterol treatment ( $p < 0.01$ ). There was no significant difference in

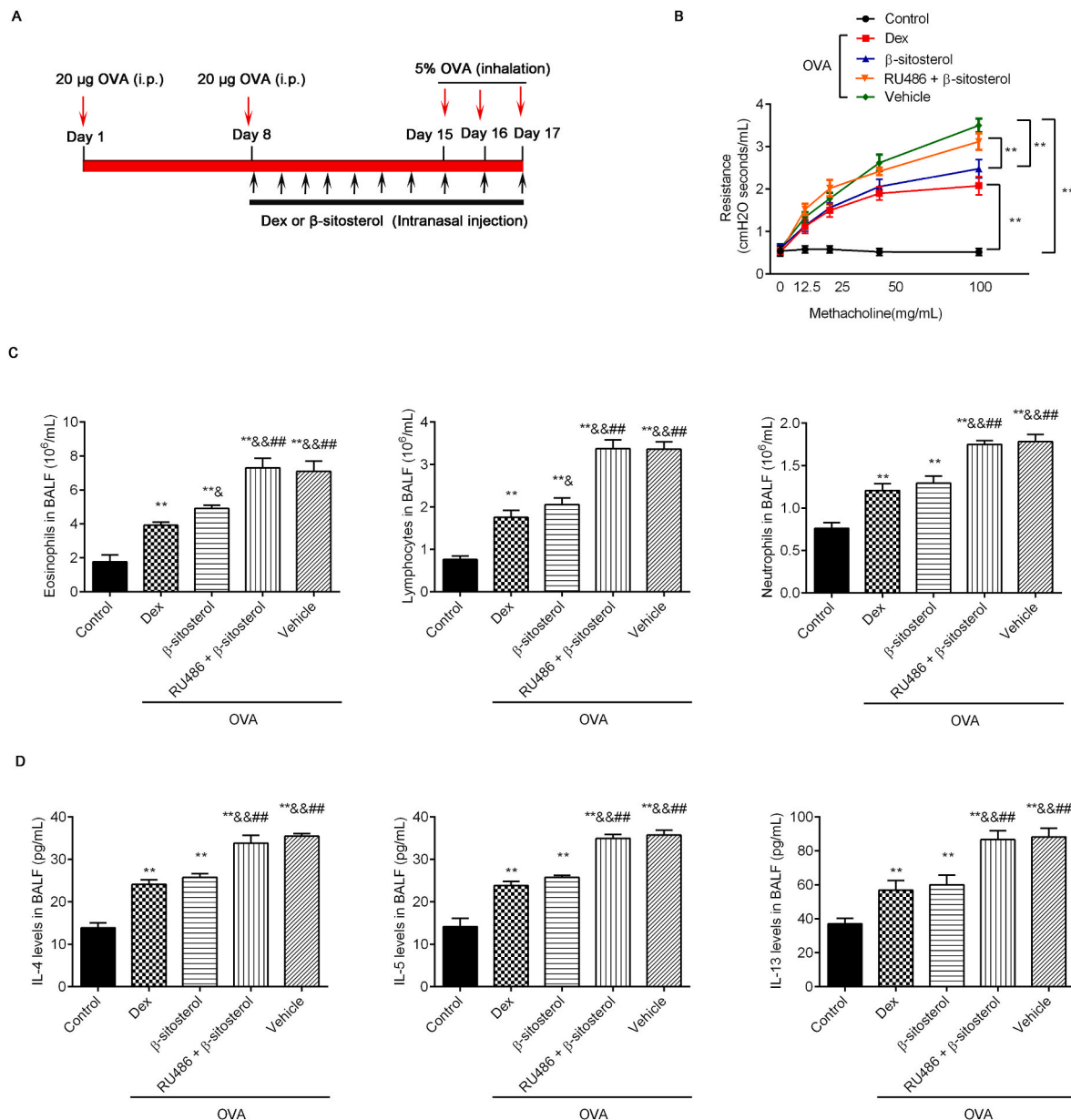


**Fig. 5.** β-sitosterol inhibited Th2 inflammation in OVA-induced mice. (A) Diagram of experimental protocols in OVA-challenged mice. Dex (2.5 mg/kg) or β-sitosterol (2.5 mg/kg) solutions used for intranasal treatment, and RU486 (20 mg/kg) was used for oral treatment to analyze the mechanism of β-sitosterol. (B) Airway hyperresponsiveness (AHR) was determined by airway resistance to increasing concentrations of nebulized methacholine.  $^{***}p < 0.01$ . (C) Numbers of eosinophils, lymphocytes and neutrophils in bronchioalveolar lavage fluids (BALFs) were analyzed using the Wright stain solution. (D) Levels of IL-4, IL-5 and IL-33 in BALFs were analyzed using ELISA. Eight parallel experiments were performed. vs. control group,  $^{*}p < 0.01$ ; vs. Dex group,  $^{&}p < 0.05$ ,  $^{&}p < 0.01$ ; vs. β-sitosterol group,  $^{##}p < 0.01$ .

the collagen fibers areas between the Dex and β-sitosterol groups. Compared with the β-sitosterol treatment, RU486 plus β-sitosterol treatment significantly increased the areas of collagen fibers in the lung tissues ( $p < 0.01$ ).

### 3.7. β-sitosterol upregulated GR expression in lung tissue of OVA-induced mice

The GR expression levels in lung tissue of each group were analyzed by immunofluorescence (Fig. 7A) and western blot (Fig. 7B). The GR expression levels were notably downregulated in the lung tissue of OVA-challenged mice, and its levels were upregulated due to Dex or β-sitosterol administration ( $p < 0.01$ ). There was no significant difference in the GR expression between the Dex and β-sitosterol treatments. The

treatment of RU486 significantly inhibited the β-sitosterol-promoted GR expression ( $p < 0.01$ ).

### 3.8. β-sitosterol suppressed fibrosis-related proteins in lung tissue of OVA-induced mice

Fibrosis-related proteins, i.e., collagen 1A, α-SMA, and TGF-β1, in the lung tissues were measured by western blot (Fig. 8A). Their relative expression levels were obviously increased in the OVA-induced lung tissues contrasted to the control lung tissues (Fig. 8B–D). In contrast to the vehicle treatment, the Dex or β-sitosterol treatment clearly suppressed the expression of these proteins in the damaged lung tissues ( $p < 0.01$ ). The expressions of α-SMA and TGF-β1 were higher in the β-sitosterol group than those in the Dex group ( $p < 0.05$ ), but no

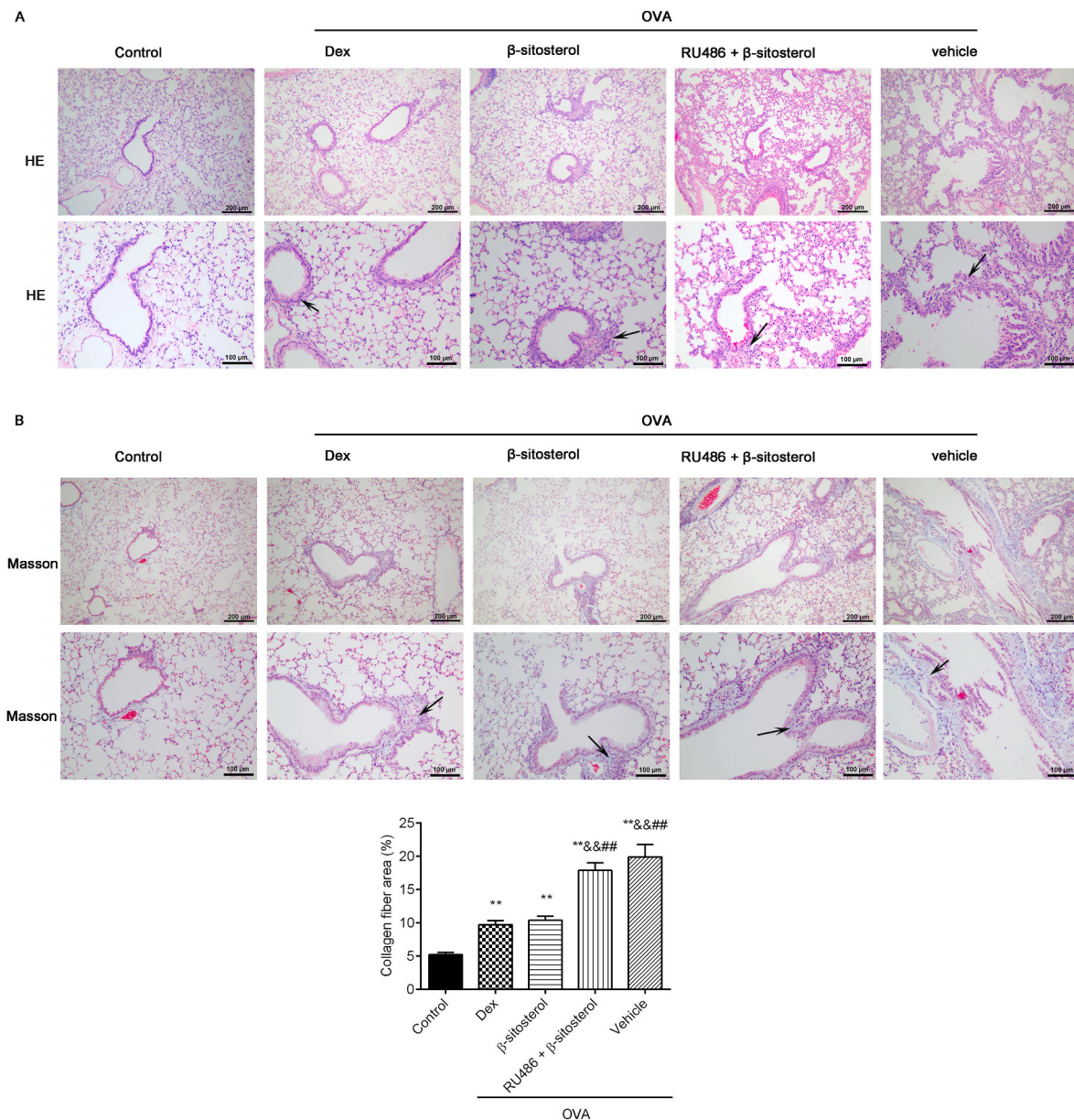


**Fig. 6.**  $\beta$ -sitosterol improved airway inflammation and fibrosis in lung tissue of OVA-induced mice. (A) Inflammation infiltration (black arrow) in lung tissue was observed by HE staining, scale = 200  $\mu$ m, 100  $\mu$ m; (B) Collagen fibers (black arrow) in lung tissue were observed Masson, scale = 200  $\mu$ m, 100  $\mu$ m. The collagen fiber area was analyzed using Image Pro Plus 6.0 software. Four parallel experiments were performed. vs. control group, \*\* $p < 0.01$ ; vs. Dex group, && $p < 0.01$ ; vs.  $\beta$ -sitosterol group, ## $p < 0.01$ .

significant difference in terms of collagen 1A expression between the two groups was identified. The application of RU486 significantly prevented the roles of  $\beta$ -sitosterol on collagen 1A and TGF- $\beta$ 1 expressions, but not  $\alpha$ -SMA expression ( $p < 0.01$ ).

#### 4. Discussion

In this study, we found good binding of  $\beta$ -sitosterol to GR (score =  $-8.2$  kcal/mol). Additionally, the GR expression was upregulated with  $\beta$ -sitosterol dose increase in TGF- $\beta$ 1-induced BEAS-2B and HBSMC, indicating that  $\beta$ -sitosterol might be an alternative to Dex in the treatment of asthma. Similar to Dex,  $\beta$ -sitosterol could fit into the binding pocket of GR and occupy the active site. Two subtypes, GR $\alpha$  and GR $\beta$ , are included in the GR, and glucocorticoids mainly bind GR $\alpha$  to exert their biological effects [24]. Due to the similar structure between Dex and  $\beta$ -sitosterol, a hydrogen bond between  $\beta$ -sitosterol and GR $\alpha$  might be its

mechanism of action.

To confirm this hypothesis, 20  $\mu$ g/mL  $\beta$ -sitosterol was selected to treat TGF- $\beta$ 1-induced BEAS-2B and HBSMC. As a phytosterol,  $\beta$ -sitosterol has been reported in OVA-induced mice, and its anti-type II inflammation has also been found [13]. Airway epithelial cells would secrete IL-25 and IL-33 to activate natural immune cells, initiating a type II immune response [2,25]. In this study, we used BEAS-2B cells to analyze  $\beta$ -sitosterol mechanism of action in airway epithelial cells. The secretions of IL-25 and IL-33 were significantly decreased by  $\beta$ -sitosterol in the TGF- $\beta$ 1-induced BEAS-2B. The results were similar to that with Dex treatment. Furthermore, GR antagonist (RU486) treatment clearly hampered the therapeutic effects of  $\beta$ -sitosterol in TGF- $\beta$ 1-induced BEAS-2B. RU486 is a GR antagonist with high affinity for both GR and progesterone receptor (PR) and a strong *in vitro* and *in vivo* GR antagonist activity [16]. The results were similar to that with Dex treatment. Furthermore, GR antagonist (RU486) treatment clearly hampered the

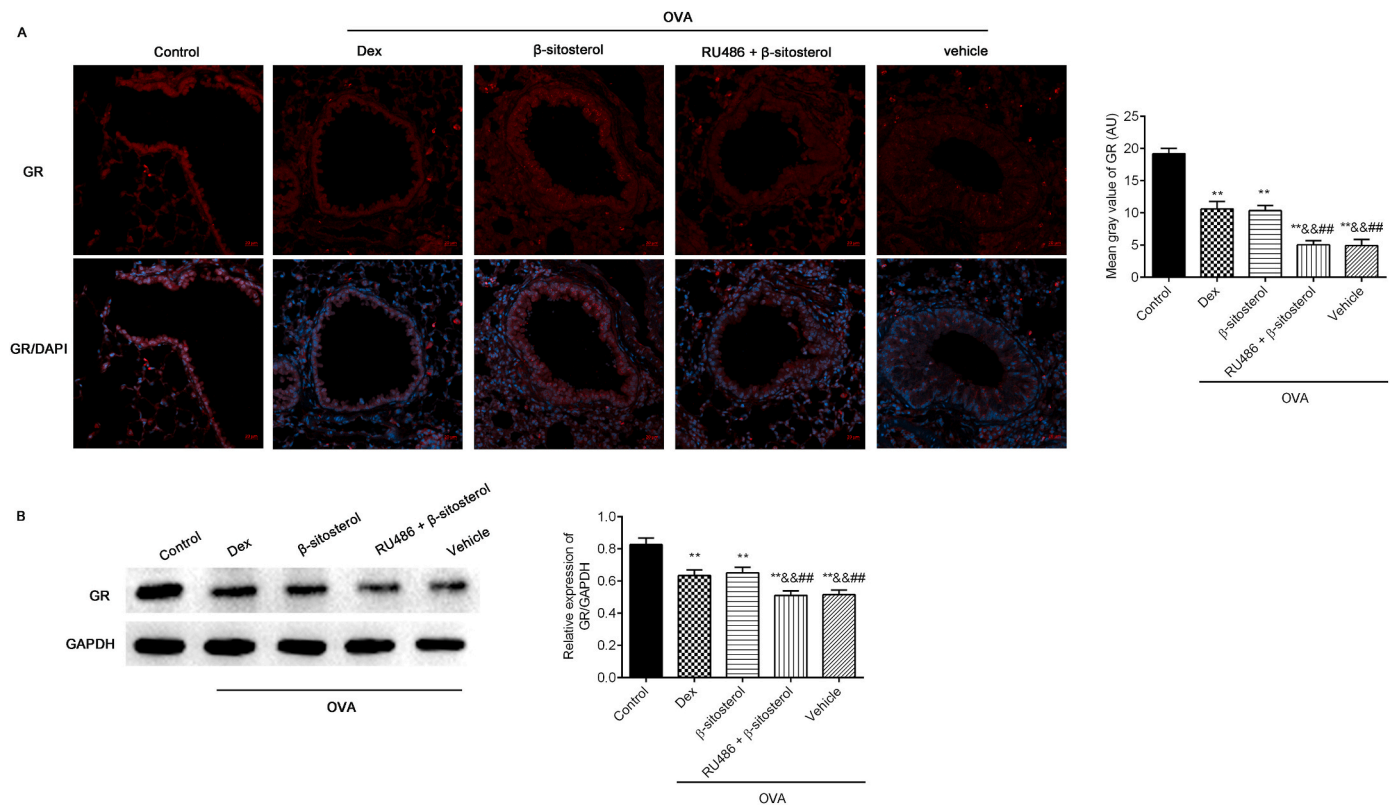


**Fig. 7.**  $\beta$ -sitosterol upregulated GR expression in lung tissue of OVA-induced mice. The GR expression in lung tissue was measured by immunofluorescence (A) and western blot (B). The mean gray value of GR and relative expression of GR were analyzed using Image Pro Plus 6.0 software. Four parallel experiments were performed. vs. control group, \*\* $p < 0.01$ ; vs. Dex group, &# $p < 0.01$ ; vs.  $\beta$ -sitosterol group, ## $p < 0.01$ .

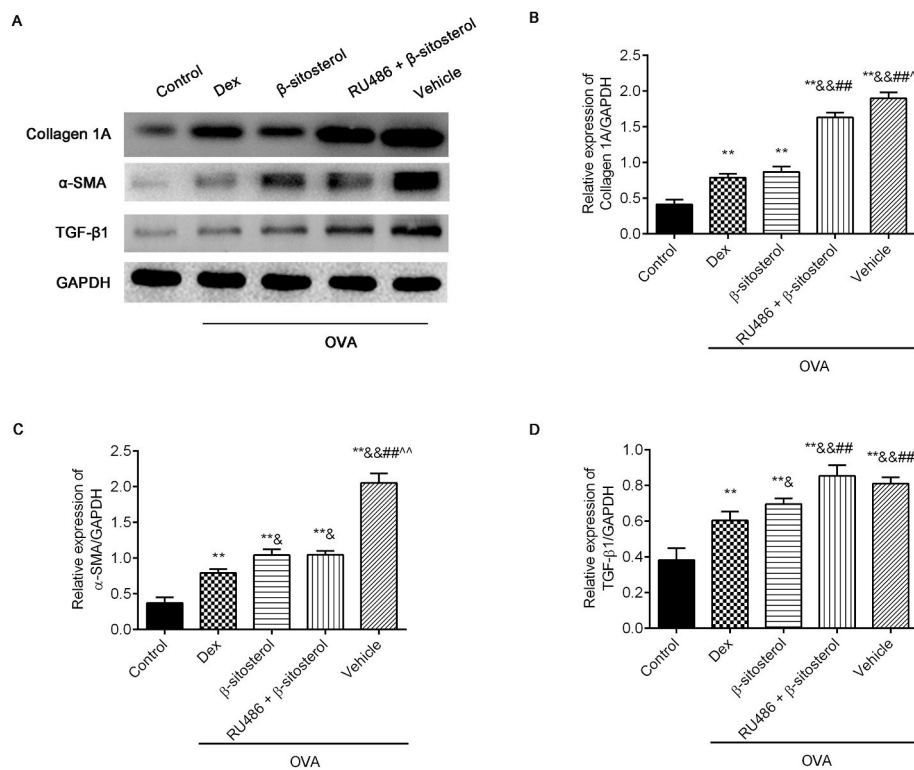


**Fig. 8.**  $\beta$ -sitosterol suppressed fibrosis-related proteins in lung tissue of OVA-induced mice. (A) The collagen 1A,  $\alpha$ -SMA and TGF- $\beta$ 1 proteins expression were measured by western blot; The relative expression of collagen 1A (B),  $\alpha$ -SMA (C) and TGF- $\beta$ 1 (D) was analyzed by Image Pro Plus 6.0 software. Four parallel experiments were performed. vs. control group, \*\* $p < 0.01$ ; vs. Dex group, &# $p < 0.05$ , &# $p < 0.01$ ; vs.  $\beta$ -sitosterol group, ## $p < 0.01$ ; vs. RU486+ $\beta$ -sitosterol group,  $\sim$  $p < 0.05$ ,  $\sim$  $p < 0.01$ .

therapeutic effects of  $\beta$ -sitosterol in TGF- $\beta$ 1-induced BEAS-2B. These results suggest that  $\beta$ -sitosterol might be a therapeutic drug for the treatment of type II inflammation and binding GR to perform its functions might be one of its mechanisms of action.

In previous studies, researchers have only shown the anti-inflammation of  $\beta$ -sitosterol in asthma [10,13], but its effects on airway remodeling have not been studied. Airway remodeling is characterized by structural changes and activation of airway smooth muscle cells and fibroblasts [26–28]. Although dysfunctional epithelial cells are involved in the structural changes of the airways, airway smooth muscle cells are the key features in varying levels of airway fibrosis. Stretching of airway smooth muscle cells results in increased TGF- $\beta$ 1 signaling and induces airway smooth muscle cell hypertrophy [28]. The TGF- $\beta$ 1 signaling activation contributes to the synthesis of matrix molecules in airway smooth muscle cells, especially collagen, fibronectin and proteoglycans [5]. In our study, the expression levels of collagen 1A and  $\alpha$ -SMA were significantly suppressed by  $\beta$ -sitosterol in the TGF- $\beta$ 1-induced HBSMC. In addition, the RU486 treatment blocked the effects of  $\beta$ -sitosterol in TGF- $\beta$ 1-induced HBSMC. These results suggest that  $\beta$ -sitosterol might be a therapeutic drug for the treatment of airway remodeling, and binding GR to perform its functions might be one of mechanisms of action.

To confirm our findings *in vitro*, the therapeutic mechanism of  $\beta$ -sitosterol administration in OVA-challenged mice was observed. The data showed that  $\beta$ -sitosterol treatment significantly improved Th2-driven inflammation and airway remodeling by suppressing type II immune response and collagen deposition. The therapeutic effects were similar to the Dex treatment. *In vivo*, the application of RU486 also significantly weakened the effects of  $\beta$ -sitosterol. The results of this study revealed that  $\beta$ -sitosterol might be a therapeutic drug for allergic asthma, which might become be an alternative to Dex in the further.

Currently, new candidates from natural resources are of research interest to find new alternative therapeutics. Studies have shown that  $\beta$ -sitosterol interferes with multiple cells signaling pathways and it has been discovered during pharmacological screening without significant toxicity [29,30]. In our study, we found that 50  $\mu$ g/mL  $\beta$ -sitosterol significantly decreased the cell viability, suggesting that we should select an appropriate concentration in the application itself. However, this research related to  $\beta$ -sitosterol remains limited. More studies are required on the metabolism, bioavailability and safety of  $\beta$ -sitosterol to support its application in the asthma treatment.

## 5. Conclusions

This study identified that new candidates from natural resources are of research interest to find new alternative therapeutics binds GR to perform its functions in asthma treatment. Through the upregulation of GR,  $\beta$ -sitosterol administration prevented Th2-driven inflammation and airway remodeling in TGF- $\beta$ 1-induced cells (BEAS-2B and HBSMC) and OVA-induced mice. These findings provide a promising therapeutic drug for allergic asthma, which might become an alternative of Dex in the future treatment of asthma.

## Statement of ethics

This study approval was reviewed and approved by the Institutional Animal Care and Use Committees of Affiliated Yantai Yuhuangding Hospital, Medical College of Qingdao University (No.2022-111).

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## Author statement

Jianfeng Xu and Lei Yang: Conceptualization, Writing-Original draft preparation, Methodology, Software. Tiantian Lin: Data curation, Visualization, Investigation. Jianfeng Xu: Supervision, Data curation, Validation. All contributing authors reviewed, edited and agreed to the final version of the manuscript.

## Declaration of competing interest

The authors have no conflicts of interest to declare.

## Data availability

Data will be made available on request.

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## References

- [1] N. Akar-Ghbiril, T. Casale, A. Custovic, W. Phipatanakul, Allergic endotypes and phenotypes of asthma, *J. Allergy Clin. Immunol. Pract.* 8 (2) (2020) 429–440.
- [2] T. Nabe, Steroid-resistant asthma and neutrophils, *Biol. Pharmaceut. Bull.* 43 (1) (2020) 31–35.
- [3] T.L. Jones, D.M. Neville, A.J. Chauhan, Diagnosis and treatment of severe asthma: a phenotype-based approach, *Clin. Med.* 18 (Suppl 2) (2018) s36–s40.
- [4] X. Li, P. Song, Y. Zhu, H. Lei, K.Y. Chan, H. Campbell, et al., The disease burden of childhood asthma in China: a systematic review and meta-analysis, *Journal of global health* 10 (1) (2020), 010801.
- [5] N. Wang, D. Yan, Y. Liu, Y. Liu, X. Gu, J. Sun, et al., A HuR/TGF- $\beta$ 1 feedback circuit regulates airway remodeling in airway smooth muscle cells, *Respir. Res.* 17 (1) (2016) 117.
- [6] M. Yang, J. Chen, W. Wei, Dimerization of glucocorticoid receptors and its role in inflammation and immune responses, *Pharmacol. Res.* 166 (2021), 105334.
- [7] Y. Leng, Y. Sun, C. Lv, Z. Li, C. Yuan, J. Zhang, et al., Characterization of  $\beta$ -sitosterol for potential selective GR modulation, *Protein Pept. Lett.* 28 (3) (2021) 276–281.
- [8] L. Pujols, J. Mullol, A. Torrego, C. Picado, Glucocorticoid receptors in human airways, *Allergy* 59 (10) (2004) 1042–1052.
- [9] C.D. Zappia, A. Soto, G. Granja-Galeano, I. Fenoy, N. Fernandez, C.A. Davio, et al., Azelastine potentiates antiasthmatic dexamethasone effect on a murine asthma model, *Pharmacol. Res. Perspect.* 7 (6) (2019), e00531.
- [10] S.G. Mahajan, A.A. Mehta, Suppression of ovalbumin-induced Th2-driven airway inflammation by  $\beta$ -sitosterol in a Guinea pig model of asthma, *Eur. J. Pharmacol.* 650 (1) (2011) 458–464.
- [11] B.X. Zhou, J. Li, X.L. Liang, X.P. Pan, Y.B. Hao, P.F. Xie, et al.,  $\beta$ -sitosterol ameliorates influenza A virus-induced proinflammatory response and acute lung injury in mice by disrupting the cross-talk between RIG-I and IFN/STAT signaling, *Acta Pharmacol. Sin.* 41 (9) (2020) 1178–1196.
- [12] Y.J. Park, I.J. Bang, M.H. Jeong, H.R. Kim, D.E. Lee, J.H. Kwak, et al., Effects of  $\beta$ -sitosterol from corn silk on TGF- $\beta$ 1-induced epithelial-mesenchymal transition in lung alveolar epithelial cells, *J. Agric. Food Chem.* 67 (35) (2019) 9789–9795.
- [13] J.E. Yuk, J.S. Woo, C.Y. Yun, J.S. Lee, J.H. Kim, G.Y. Song, et al., Effects of lactose-beta-sitosterol and beta-sitosterol on ovalbumin-induced lung inflammation in actively sensitized mice, *Int. Immunopharm.* 7 (12) (2007) 1517–1527.
- [14] E.F. Pettersen, T.D. Goddard, C.C. Huang, G.S. Couch, D.M. Greenblatt, E.C. Meng, et al., UCSF Chimera—a visualization system for exploratory research and analysis, *J. Comput. Chem.* 25 (13) (2004) 1605–1612.
- [15] T.D. LeVan, E.A. Babin, H.I. Yamamura, J.W. Bloom, Pharmacological characterization of glucocorticoid receptors in primary human bronchial epithelial cells, *Biochem. Pharmacol.* 57 (9) (1999) 1003–1009.
- [16] F. Spiga, D.M. Knight, S.K. Droste, B. Conway-Campbell, Y. Kershaw, C. P. MacSweeney, et al., Differential effect of glucocorticoid receptor antagonists on glucocorticoid receptor nuclear translocation and DNA binding, *J. Psychopharmacol.* 25 (2) (2011) 211–221.
- [17] D.I. Kim, M.K. Song, K. Lee, Comparison of asthma phenotypes in OVA-induced mice challenged via inhaled and intranasal routes, *BMC Pulm. Med.* 19 (1) (2019) 241.
- [18] D. Masid-de-Brito, B.M. Vieira, C.C. de Souza, F. Silva, M.I.C. Gaspar-Elsas, P. Xavier-Elsas, Allergen challenge-induced changes in bone-marrow responses to leukotriene D(4), nonsteroidal anti-inflammatory drugs and cytokines, *Immunopharmacol. Immunotoxicol.* 42 (3) (2020) 199–210.
- [19] M. Verma, S. Liu, L. Michalec, A. Sripada, M.M. Gorska, R. Alam, Experimental asthma persists in IL-33 receptor knockout mice because of the emergence of thymic stromal lymphopoietin-driven IL-9(+) and IL-13(+) type 2 innate lymphoid cell subpopulations, *J. Allergy Clin. Immunol.* 142 (3) (2018) 793–803, e8.

- [20] X. Zhou, B.B. Moore, Lung section staining and microscopy, *Bio-protocol* 7 (10) (2017).
- [21] J.G. Donaldson, Immunofluorescence Staining. *Current Protocols in Cell Biology*, 2001 (Chapter 4):Unit 4.3.
- [22] K.H. Mohan, S. Pai, R. Rao, H. Sripathi, S. Prabhu, Techniques of immunofluorescence and their significance, *Indian J. Dermatol., Venereol. Leprol.* 74 (4) (2008) 415–419.
- [23] C. Lee, Western blotting, *Methods Mol. Biol.* 362 (2007) 391–399.
- [24] L. Pujols, J. Mullol, C. Picado, Importance of glucocorticoid receptors in upper and lower airways, *Front. Biosci.* 15 (2) (2010) 789–800.
- [25] T. Boonpiyathad, Z.C. Sözen, P. Satitsuksanoa, C.A. Akdis, Immunologic mechanisms in asthma, *Semin. Immunol.* 46 (2019), 101333.
- [26] N. Hirota, J.G. Martin, Mechanisms of airway remodeling, *Chest* 144 (3) (2013) 1026–1032.
- [27] B. Camoretti-Mercado, R.F. Lockey, Airway smooth muscle pathophysiology in asthma, *J. Allergy Clin. Immunol.* 147 (6) (2021) 1983–1995.
- [28] A. Banno, A.T. Reddy, S.P. Lakshmi, R.C. Reddy, Bidirectional interaction of airway epithelial remodeling and inflammation in asthma, *Clin. Sci. : 1979* 134 (9) (2020) 1063–1079.
- [29] Z. Khan, N. Nath, A. Rauf, T.B. Emran, S. Mitra, F. Islam, et al., Multifunctional roles and pharmacological potential of  $\beta$ -sitosterol: emerging evidence toward clinical applications, *Chem. Biol. Interact.* 365 (2022), 110117.
- [30] M.S. Bin Sayeed, S.S. Ameen, Beta-sitosterol: a promising but orphan nutraceutical to fight against cancer, *Nutr. Cancer* 67 (8) (2015) 1214–1220.