

Anthocyanins Inhibit Airway Inflammation by Downregulating the NF- κ B Pathway via the miR-138-5p/SIRT1 Axis in Asthmatic Mice

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Keywords

Anthocyanins · Asthma · miR-138-5p · Sirtuin-1 · Nuclear factor- κ B · Inflammatory factors · Anti-inflammatory · Airway hyperresponsiveness

Abstract

Objective: Asthma, caused by chronic inflammation, is a common disease. Anthocyanins are involved in asthma treatment. This study explored the mechanism of anthocyanins on airway inflammation in asthmatic mice by regulating nuclear factor- κ B (NF- κ B) via the miR-138-5p/sirtuin-1 (SIRT1) axis. **Methods:** The asthmatic mouse model was established by ovalbumin (OVA) induction and treated with anthocyanins or simultaneously injected with the lentivirus miR-138-5p mimic, followed by the measurement of lung inflammatory injury and IL-4, IL-5, IL-13, and IFN- γ levels in bronchoalveolar lavage fluid. Human bronchial epithelial (HBE) cells 16HBE14o-¹⁶⁰ were induced by OVA to establish an asthmatic cell model, treated with anthocyanins and manipulated with miR-138-5p mimic and pcDNA3.1-SIRT1. The releases of inflammatory cytokines, the nuclear translocation of p-p65/p65 in the NF- κ B pathway, and the levels of miR-138-5p and SIRT1 mRNA were detected. **Results:** In vivo experiments showed that anthocyanins could reduce the OVA-induced

airway hyperresponsiveness and airway inflammation, improve the inflammatory infiltration and mucus in lung tissues, and diminish the miR-138-5p level in asthmatic mice. Infection with the miR-138-5p mimic averted the remission effect of anthocyanins in asthmatic mice. In vitro experiments showed that in HBE cells exposed to OVA, anthocyanins reduced the miR-138-5p level, increased the SIRT1 level, inhibited the release of inflammatory factors, and reduced the nuclear translocation of NF- κ B p65. miR-138-5p targeted SIRT1. miR-138-5p overexpression partially reversed the therapeutic effect of anthocyanins, while SIRT1 overexpression antagonized the effect of miR-138-5p overexpression. **Conclusion:** Anthocyanins inhibited the NF- κ B pathway by regulating the miR-138-5p/SIRT1 axis, thus inhibiting airway inflammation in asthmatic mice.

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Introduction

Asthma is a chronic disorder of the airway inflammatory, which is principally characterized by cough, breathlessness, and an episodic wheeze, caused by airway in-

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flammation and hyperresponsiveness [1]. It is estimated to influence about 300 million people all over the world, with a significantly increasing prevalence [2, 3]. Although the pathogenesis of asthma has not been fully understood, the risk factors of asthma have been identified, which include epigenetic variations, respiratory infections, airborne environmental exposures, sensitization to inhalant allergens and atopic conditions, effects of vitamin D, microbiome, chemical exposure, metabolites, and stress [4]. However, generally speaking, asthma is difficult to treat as a heterogeneous disorder [5]. As asthma has long been predominately identified as an inflammatory condition, the current therapeutic interventions mainly primarily focus on alleviating inflammation [6].

Anthocyanins are one of the 6 subfamilies of a widespread and large group of the plant constituents identified as flavonoids, which are responsible for the attractive and bright red, orange, blue, and purple colors of most flowers, vegetables, fruits, and some cereal grains [7]. As anthocyanins are commonly consumed, their biological activities have been extensively noticed and studied, which are found to have anti-inflammatory, anti-oxidative, antimutagenic, and antimicrobial properties that play a role in the prevention and treatment of a variety of chronic diseases [8]. It is reported that flavonoids, like anthocyanins, can reduce cyclooxygenase gene activation, which is crucial in the inflammatory response, and their products are important in the pathogenesis of inflammatory diseases, including asthma [9, 10].

MicroRNAs (miRNAs) are a series of 19–22-nucleotide single-stranded noncoding RNAs, which inhibit the translation of genes and downregulate target gene expressions by binding to the 3'-untranslated regions (3'-UTRs) of mRNAs [11]. miRNAs are known to play roles in a variety of biological processes, such as development, differentiation, regulation, and stress of inflammation in the host immune cells and are reported to be the master regulators of the pathways involved in asthma [3]. Anthocyanins attenuate inflammation in human corneal epithelial cells through regulating miRNAs [12]. miR-138 is reported to regulate the balance of Th1/Th2 and alleviate inflammatory response in asthma [13]. The pathological effect of the cellular senescence involving the silent information regulator 2 homolog sirtuin (SIRT) protein family has been shown in exacerbated and stable chronic respiratory diseases, especially in asthma [14]. Nuclear factor- κ B (NF- κ B) transferred from the cytoplasm to the nucleus initiates gene transcriptions such as chemokines, inflammatory mediators, and inflammatory cytokines [15]. The activation of the NF- κ B pathway plays an es-

sential role in inflammation-related diseases, such as asthma [16]. Anthocyanins exert the anti-inflammatory effect through the modulation of the NF- κ B signaling cascade [17]. However, there is no domestic and foreign report at present on whether anthocyanins regulate NF- κ B expression through miR-138-5p, thus inhibiting airway inflammation in asthmatic mice. This study set out to investigate the mechanism of anthocyanins on inhibiting the airway inflammation in asthma, so as to provide a new theoretical basis for the treatment of asthma.

Materials and Methods

Ethics Statement

All procedures were authorized by the laboratory animal Ethics Committee. All procedures were strictly implemented by the Guide for the Care and Use of Laboratory Animals. All the laboratory procedures were used to reduce the pain of the mice.

Anthocyanins

Anthocyanins, purchased from Huich (HA11386; Shanghai, China), were the natural extracted anthocyanins standard, with the effective component of cyanidin-3-O glucoside. The purity of the product determined using the high-performance liquid chromatography method was $\geq 98\%$ [18].

Model Establishment

A total of 70 male Balb/c mice (7–8 weeks old, weighing 18 ± 5 g) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (SCXK [Shanghai] 2017-0005; Shanghai, China). The mice were raised for 1 week in 12-h light/dark cycles at a constant temperature ($23^\circ\text{C} \pm 3^\circ\text{C}$) with a relative humidity ($50\% \pm 10\%$). The mice were assigned into the following 7 groups on average according to random numbers as follows: (1) normal group (on days 0 and 7, the mice were intraperitoneally injected with 500 μL normal saline, and on days 14–20, the mice were placed in a box and nebulized with normal saline using an ultrasonic atomizer [Omron, Tokyo, Japan] for 30 min every day); (2) asthma induction model group (ovalbumin [OVA]) (on day 0 and 7, the mice were intraperitoneally injected with a 500 μL normal saline mixture containing 20 μg OVA [Sigma-Aldrich, St. Louis, MO, USA] and 1 mg aluminum hydroxide (Sigma-Aldrich) for sensitization; on days 14–20, the mice were exposed to an atomization environment, consisting of normal saline containing 5% OVA for 30 min every day to stimulate allergies); (3) anthocyanin intervention group (OVA + Anth) (the treatment was the same as the OVA group but 250 mg/kg anthocyanin was administered to the mice 1 h before each atomization [19]); (4) miR-138-5p overexpression group (OVA + Anth + miR mimic) (the treatment was the same as the OVA + Anth group, but on day 7, when sensitized, the mice were infected with 30 μL lentivirus solution of the miR-138-5p mimic lentiviral vector [1×10^8 PFU/mL; GenePharma, Shanghai, China]); (5) OVA + Anth + mimic NC group (the treatment was the same as the OVA + Anth + miR mimic group, but a simulant negative control lentivirus vector was used for transfection); (6) co-intervention negative control group (OVA + Anth + miR mimic + oe NC) (the treatment was the same as the OVA + Anth group, but

on day 7, when sensitized, the mice were infected with 30 μ L miR-138-5p lentivirus vector and 30 μ L pcDNA3.1 lentivirus vector solution [GenePharma] from the tail vein at a virus titer of 1×10^8 PFU/mL; (7) co-intervention group (OVA + Anth + miR mimic + oe SIRT1) (the treatment was the same as the OVA + Anth + miR mimic + oe NC group, but the mice were injected with pcDNA3.1-SIRT1 lentivirus vector solution).

Airway Hyperresponsiveness Determination

After 24 h of the last stimulation, the mice were anesthetized with pentobarbital sodium (90 mg/kg). The trachea was exposed and cannulated to the pulmonary function instrument of small animals (flexiVent; Scireq, Montréal, Canada), and the instrument was closed. The respiratory rate was adjusted to 90 times/min, with a tidal volume of 200 mL. Different doses of acetylcholine (0, 6.25, 12.5, 25, and 50 μ g/kg) were given through the tail vein. The maximum value of lung resistance was recorded after administration using the Jaeger Masterscreen Body volumetric recorder system (Hoechst, Germany).

Sample Collection

When airway hyperresponsiveness (AHR) was detected, 0.2 mL blood sample was immediately collected from the mouse orbit, centrifuged at 3,500 rpm for 10 min, and the serum was separated and stored at -20°C . Then, the mice were euthanized by pentobarbital sodium (i.p., 200 mg/kg). The mice were fixed in a supine position, and the trachea of the mice was exposed. Normal saline was injected using an intravenous syringe to flush the lungs for 3 times, and the bronchoalveolar lavage fluid (BALF) samples were collected and centrifuged, and the supernatant was stored at -80°C for measurement of cytokine levels. After BALF collection, the lung tissues of each mouse were collected, fixed in a buffer containing 10% formalin, paraffin-embedded in paraffin, and sliced into 5- μ m sections.

Detection of Inflammatory Cells in BALF

The BALF cells were resuspended in phosphate-buffered saline (PBS). Cell smears were made by cell precipitation and naturally dried to 70%. The samples were stained with Wright-Giemsa (Bio-Vision, Milpitas, CA, USA). The cells were counted and classified under an optical microscope (Olympus, Tokyo, Japan; BX53, $\times 40$ magnification) using the improved Neubauer counting board. The numbers of eosinophil (orange), macrophage (dusty blue), lymphocyte (bluish violet), and neutrophil (lavender) in BALF were measured using a hemocytometer under an optical microscope ($\times 400$ magnification).

Hematoxylin and Eosin Staining

The slides of paraffined sections were placed on the staining rack. The samples were cleared with xylene for 3 times, 2 min for each time; soaked in 100% (twice), 95%, and 70% alcohol for 2 min; and washed with distilled water for 2 min. The sample tissues were stained in hematoxylin solution for 3 min, placed in distilled water for 5 min, stained with eosin dye for 2 min, washed, dehydrated with 95% and 100% alcohol, washed in xylene for 3 times, and placed under an optical microscope (Olympus; BX53) for observation.

Alcian Blue-Periodic Acid-Schiff Staining

The paraffined sections of mouse lung tissue were dewaxed in distilled water, oxidized in 0.5% periodate solution for 5 min, washed with tap water, and washed with distilled water. The sec-

tions were stained in 1% alcian blue solution for 5–10 min and washed with tap water and distilled water. The sections were stained in the reagent for 10–20 min, washed with tap water, and then soaked in distilled water for 10 min and dehydrated with alcohol, cleared with xylene, and sealed with neutral gum. The sections were analyzed in the color image analysis system. Lung tissue section morphological identification was performed as the following steps: the stained sections were scanned and photographed using Olympus VS120 (Olympus; $\times 20$ magnification). The images were processed using OlyVIA 2.6 software (Olympus Soft Imaging Solutions GmbH) and measured and recorded using NIS-Elements Basic software (BR 3.2 64; Nikon).

The scoring parameters for inflammatory infiltration analysis were as follows: 0 presented that there was no infiltration in the parenchymal or peripheral airway; 1 presented sparse infiltration (0–20% of airway and vascular infiltration <50% of circumference); 2 presented moderate infiltration (0–20% of airway and vascular infiltration >50% of circumference); 3 presented extensive infiltration (20–70% of the large airways were surrounded by cell infiltration); 4 presented large infiltration (70–100% of the large airway and small airway were surrounded by infiltration, and the infiltrating area is far away from the airway/blood vessel). As for mucus-secreting cell counting, in periodic acid-Schiff (PAS) staining, blue/purple goblet cells were identified as PAS-positive. The numbers of PAS-negative and PAS-positive cells around the airway were counted. The PAS-positive goblet cells were expressed as the number of PAS-positive cells in per 100 μ m basement membrane. Morphological analysis was performed to analyze the volume density of PAS-positive substance in lobar bronchus and segmental bronchus using the Image J software (NIH, Bethesda, MD, USA). The above tests were conducted using a double-blind method.

Cell Culture and Treatment

Human bronchial epithelial (HBE) cells (16HBE14 $_{\text{o}}$ ¹⁶⁰) were purchased from the China National Center cell bank. The cells were cultured in DMEM (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C in a humidified incubator containing 5% CO_2 . The 16HBE14 $_{\text{o}}$ ¹⁶⁰ cells were assigned into the following groups: blank group, OVA group, OVA + Anth group, OVA + Anth + miR mimic NC group, OVA + Anth + miR-138-5p mimic group, OVA + Anth + miR-138-5p mimic + pcDNA3.1 group, and OVA + Anth + miR-138-5p mimic + pcDNA3.1-SIRT1 group. The cells were cultured in 6-well plates, and when the density reached 70%, cells were treated accordingly. The cells in the blank group were normally cultured, and the cells in other groups were added with 50 μ m/L OVA and treated for 48 h. After 48 h, the cells in the Anth intervention groups were added with 10 μ g/mL anthocyanins and treated for 1 h [20], and the corresponding experiments were performed. The miR-138-5p mimic vector, pcDNA3.1-SIRT1, and the corresponding negative controls were synthesized by GenePharma. The cells were seeded into 6-well plates at 3×10^5 cells/mL. When the cell confluence reached 40–60%, the cells were transfected. Cell transfection buffer was prepared. Tube A was cultured in 5 μ L/well Lipofectamine 2000 (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 250 μ L Opti-MEM medium (Gibco). Tube B was, respectively, cultured with the miR-138-5p mimic and negative control (5 μ L/well) in 250 μ L Opti-MEM medium. Tube A was incubated at room temperature for 5 min and mixed with tube B for 20 min. After culture for 6 h in an incubator, the cells

were incubated in a refreshed DEME (Gibco) containing 10% fetal bovine serum for 24 h, seeded into 6-well plates, and treated with OVA and anthocyanins according to the previous method.

Enzyme-Linked Immunosorbent Assay

Interleukin (IL)-4, IL-5, IL-13, IFN- γ , and OVA-specific IgG in BALF and OVA-specific IgE in mouse serum were detected using the corresponding enzyme-linked immunosorbent assay (ELISA) kits (Abcam, Cambridge, MA, USA), and the inflammatory factors IL-4, IL-5, IL-13, and IFN- γ in 16HBE14_o-160 cells were detected by ELISA. First, the standard samples were diluted, and the number of plates was determined according to the number of samples to be tested plus the number of standard samples. Subsequently, 50 μ L diluted standard, 50 μ L samples to be tested, and 50 μ L biotin-labeled antibody were added into the reaction wells immediately, and the plate was covered. The samples were gently shaken, mixed well, and incubated at 37°C for 1 h. Then, the samples were shaken and washed. Each well was added with 80 μ L avidin-HRP, and the samples were gently shaken, mixed well, and incubated at 37°C for 30 min. After washing, 50 μ L substrate A and 50 μ L substrate B were added into each well. The samples were gently shaken, mixed well, incubated at 37°C for 10 min, and added with 50 μ L termination solution to terminate the reaction. The optical density value of each well was determined at 450 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

Extraction of Nuclear and Cytoplasmic Protein

When the cells were cultured to 80–90% density, they were washed with precooled PBS. In accordance with the instructions of nuclear protein and cytoplasmic protein extraction kits (Beyotime Biotechnology, Shanghai, China), the cells were added with cytoplasmic extraction buffer A, vortex shaken for 30 s, placed on ice for 10–15 min, and shaken every 5 min. Then, the cells were added with cytoplasmic extraction buffer B, vortex shaken for 10 s, placed on ice for 1 min, and then centrifuged at 1,000 g at 4°C for 5 min. The precipitate was crude nuclear extract, and the supernatant obtained was cytoplasmic protein. The supernatant was added with 20% glycerol and stored at –70°C. Then, the precipitate was added with cytoplasmic extraction buffer A to wash the precipitation, centrifuged at 4°C at 1,000 g for 5 min, added with nuclear extraction buffer, vortex shaken for 10 s and placed on ice for 30 min, and shaken every 5 min. After that, the samples were centrifuged at 4°C at 1,000 g for 10 min. The supernatant was absorbed as the nucleoprotein and stored at –70°C.

Western Blot

Cells were collected and lysed to obtain proteins. Protein concentration was determined using the bicinchoninic acid method. Each lane was added with 20 μ g total protein sample. Protein isolation was performed on 12% SDS-polyacrylamide gel electrophoresis at 120 V for 90 min. The isolated protein was moved to polyvinylidene fluoride membranes at 250 mA for 90 min. Then, the membranes were incubated with 50 g/L TBST buffer for 1 h and sealed and added with SIRT1 antibody (1:1,000, ab189494; Abcam), NF- κ B p65 antibody (1:1,000, ab32536; Abcam), and NF- κ B p-p65 antibody (1:1,000, MA5-15160; Thermo Fisher Scientific, Inc.) at 4°C overnight. The next day, after washing, the membranes were hybridized with secondary goat anti-rabbit IgG antibody labeled with anti-biotin (1:2,000, ab6721; Abcam) and incubated at 37°C for 1 h. After washing, the membranes were added with an

enhanced chemiluminescence luminescent reagent. GelDoc software was used for image acquisition and data analysis. PCNA (1:1,000, ab16048; Abcam) was used as the loading control for nuclear NF- κ B p65 and NF- κ B p-p65, and β -actin (1 μ g/mL, ab8227; Abcam) was used as the internal reference for others.

Bioinformatics Analysis

The downstream targets of miR-138-5p were analyzed and predicted using microT (http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=MicroT_CDS/index) [21], miRanda (<http://www.microrna.org/microrna/home.do>) [22], miRmap (<https://mirmap.ezlab.org/>) [23], and TargetScan (http://www.targetscan.org/vert_72/) [23], and the intersection was obtained. The downstream target gene SIRT1 of miR-138-5p was obtained after screening. The binding sites of miR-138-5p and SIRT1 were predicted through the TargetScan.

Dual-Luciferase Assay

HEK293T cells (National Center cell bank, Beijing, China) were used for the dual-luciferase assay. First, the binding sites of miR-138-5p on SIRT1 were predicted using bioinformatics and amplified by polymerase chain reaction (PCR). Subsequently, the amplified fragment was inserted into the pmiR-GLO report vector to construct the SIRT1 wild-type plasmid (SIRT1-WT). The SIRT1-mutant (SIRT1 MUT) plasmid was constructed by mutation of some nucleotides by a gene mutation technique. After SIRT1-WT plasmid, SIRT1-MUT plasmid, and miR-138-5p mimic or mimic NC (purchased from GenePharma) were transfected into the cells, the luciferase activity of each group was detected using the luciferase test kit (Beyotime Biotechnology).

RNA Pull-Down Assay

The miR-138-5p sequence was cloned into the pcDNA3.1 vector. The primer sequences of PCR are shown in Table 1. The in vitro transcription and biotin labeling were performed using the Roche in vitro transcription kit (Roche, Indianapolis, IN, USA) after the constructed vector was digested with the BamH I enzyme. After the transcription, the samples were added with DNase I for detachment of the transcription template. TRIzol was used to extract the transcripts in vitro. After quantification, 3 μ g RNA was mixed with 16HBE14_o-160 cell extract for the RNA pull-down assay. The reaction system was 25 mM Tris-Cl (pH = 7.4), 150 mM NaCl, 0.05% NP-40, 0.5 mM DTT, and 1 $\times$ complete protease inhibitors (Roche). The samples were incubated at room temperature for 2–4 h and then added with TI beads (Roche) coupled with streptomycin and incubated for 30 min. Then, the samples were eluted for 5 times to extract the RNA. After reverse transcription, the adsorbed SIRT1 was detected by reverse-transcription quantitative PCR (RT-qPCR).

Total RNA Extraction and RT-qPCR

The total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The total RNA was reverse-transcribed into cDNA using the PrimeScript RT reagent kit (Takara, Dalian, China). According to the standard protocol of Bio-Rad PCR system (Bio-Rad), the expression of miR-138-5p and SIRT1 was determined using RT-qPCR. GAPDH was used as the internal reference for mRNA, and U6 was used as the internal reference for miRNA. The relative expression was calculated using the $2^{-\Delta\Delta CT}$ method. The primer sequences (synthesized by Shanghai Sangon Biotech Co., Ltd.) are shown in Table 1.

Table 1. Primer sequence

Gene	Forward 5'–3'	Reverse 5'–3'
<i>miR-138-5p</i> (human)	GGCCGGACTAAGTGTGT	GCAGGGTCCGAGGTATTC
<i>SIRT1</i> (human)	CTAGGACTTCTCCATACTC	CTGATAGGGTGTACGAAGGA
<i>GAPDH</i> (human)	AAGAAGGTGGTGAAGCAGGC	TCCACCACCTGTTGCTGTA
<i>U6</i> (human)	GCTTCGGCAGCACATATACTAAAT	CGCTTCACGAATTTGCGTGTCTAT
<i>SIRT1</i> (mouse)	TGATTGGCACCGATCCTCG	CCACAGCGTCATATCATCCAG
<i>miR-138-5p</i> (mouse)	AGCTGGTGTGTGAATCAGGCCG	AACGCTTCACGAATTTGCGT
<i>GAPDH</i> (mouse)	CAGGAGGCATTGCTGATGAT	AAGGCTGGGGCTCATTT
<i>U6</i> (mouse)	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT

Immunofluorescence

The 16HBE14_o-160 cells were treated according to the previous method. Subsequently, the cells were fixed, permeabilized and blocked, and incubated with the NF- κ B p65 antibody (1:100, ab32536; Abcam) at 4°C overnight. Then, the cells were rinsed with PBS, exposed to fluorescein isothiocyanate (1:100, ab223994; Abcam)-bound secondary antibody, stained with 4',6-diamidino-2-phenylindole (Sigma-Aldrich) for 30 min, and imaged using an Olympus FV1000 confocal microscope.

Data Processing

All data were processed using SPSS21.0 statistical software (USA). GraphPad Prism 8.01 (GraphPad Software Inc.) was used for mapping. The measurement data were expressed as mean $\pm$ standard deviation. The *t* test was applied for comparisons between 2 groups, and 1-way ANOVA was applied for comparisons among multigroups. Tukey's multiple comparisons test was used for the post hoc test. *p* < 0.05 indicated statistical significance.

Results

Anthocyanins Improved Airway Responsiveness and Inflammation in the Asthmatic Mouse Model

In various studies, the protective effect of anthocyanins on asthma has been confirmed [24]. The asthmatic mouse model was established by OVA induction. Compared with the normal group, the airway resistance was significantly increased in the OVA group, while anthocyanins could significantly reduce the OVA-induced airway resistance (Fig. 1a, *p* < 0.05). ELISA results demonstrated that the level of serum OVA-specific IgE was increased (Fig. 1b, *p* < 0.05); the level of IgG in BALF was increased (Fig. 1c, *p* < 0.05); the levels of IL-4, IL-5, and IL-13 in BALF were increased; and the level of IFN- γ in BALF was significantly decreased in the OVA group (Fig. 1d–g, *p* < 0.05). Blood cell counting showed that the numbers of eosinophils, lymphocytes, and neutrophils in BALF were increased in the OVA group (Fig. 1h, *p* < 0.05). Hematoxylin and eosin staining and alcian blue-

PAS staining of the lung tissue sections demonstrated that in the mouse lung tissue sections of the OVA group, the airway smooth muscle was thickened, a large amount of inflammatory-cell infiltration was found around the bronchus and blood vessels, the number of mucous cells was increased, and mucus secretion and goblet-cell hypertrophy were observed (Fig. 1i–k, *p* < 0.05). Anthocyanins could inhibit AHR and decrease the serum OVA-specific IgE level, the number of inflammatory cells, inflammatory factors, and IgG levels in BALF in asthmatic mice. Compared with the OVA group, the infiltration of inflammatory cells was significantly reduced, the airway structure was relatively complete, and the secretion of mucus and the proliferation of goblet cells were alleviated in the lung tissue sections of asthmatic mice after anthocyanin intervention (Fig. 1a–f). The results mentioned above suggested that anthocyanins could inhibit AHR and reduce airway inflammation in asthmatic mice.

Anthocyanins Downregulated the Level of miR-138-5p in Asthmatic Model Mice

miR-138-5p is associated with asthma-related inflammation [13]. Therefore, in vivo experiments were conducted to verify the regulatory effect of anthocyanins on miR-138-5p. miR-138-5p in lung tissues of mice after corresponding treatments was detected by RT-qPCR. Compared with the normal group, miR-138-5p was significantly upregulated in the OVA-induced asthma model in vivo (*p* < 0.01, Fig. 2a). Compared with the OVA group, miR-138-5p was significantly downregulated after anthocyanin intervention (*p* < 0.05). Subsequently, the mice were infected with the miR-138-5p overexpression lentivirus vector (the infection efficiency was shown in Fig. 2b) to verify the role of anthocyanins in the airway inflammation response mechanism in asthmatic mice in vivo. First, the airway reactivity, the inflammatory cells in BALF, and lung tissue section staining of mice in each group were

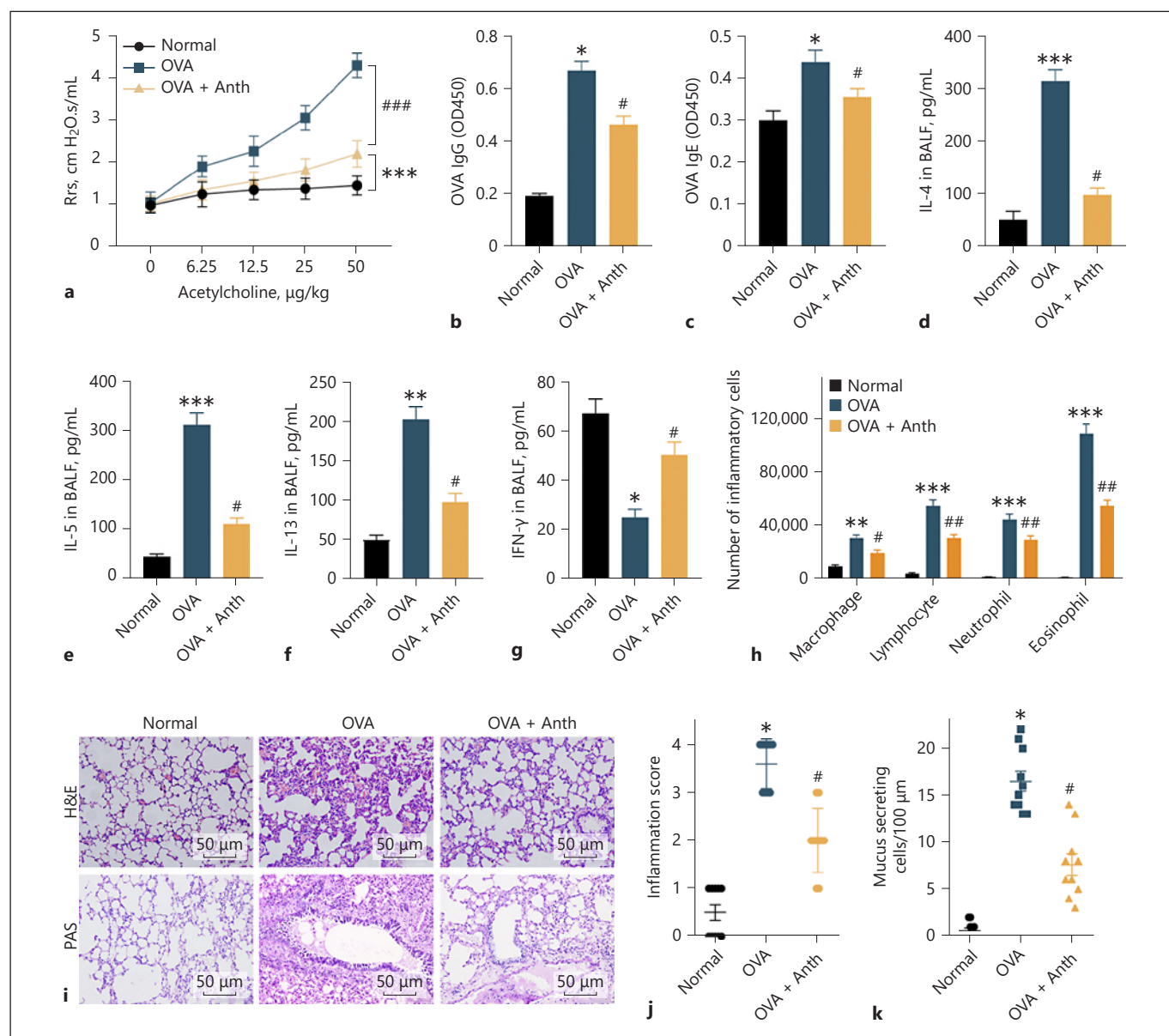


Fig. 1. Anthocyanins improved airway responsiveness and inflammation in asthmatic mice. The OVA-induced asthma model of mice was established and treated with anthocyanins. The airway response ability in mice was measured by intravenously injecting 0, 6.25, 12.5, 25, and 50 µg/kg Ach (**a**); levels of OVA-specific IgE in the serum and IgG levels in BALF were detected using ELISA kits (**b, c**); levels of IL-4, IL-5, IL-13 and IFN-γ in BALF were detected using ELISA kits (**d-g**); number of eosinophils, macrophages, lymphocytes, and neutrophils in BALF was measured using a hemocytometer (**h**); HE

staining and PAS staining were performed in the mouse lung tissues (magnification, ×400) (**i**); lung inflammation scoring and mucus secretory cell counting (**j, k**). $N = 10$. One-way ANOVA was used for data analysis. Tukey's multiple comparisons test was used for the post hoc test. Compared with the normal group, $*p < 0.05$, $***p < 0.001$; compared with the OVA group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$. Ach, acetylcholine; HE, hematoxylin and eosin; OVA, ovalbumin; BALF, bronchoalveolar lavage fluid; ELISA, enzyme-linked immunosorbent assay; PAS, periodic acid-Schiff.

measured and analyzed. Compared with the OVA + Anth + mimic NC group, the airway resistance was increased in the OVA + Anth + miR mimic group (Fig. 2c, $p < 0.001$). ELISA results showed that levels of OVA-specific IgE in

the serum and IgG in BALF were increased (Fig. 2d, e, all $p < 0.05$); the levels of IL-4, IL-5 and IL-6 were increased, the levels of IFN-γ were decreased (Fig. 2f, all $p < 0.05$); and inflammatory cells in BALF were increased (Fig. 2g,

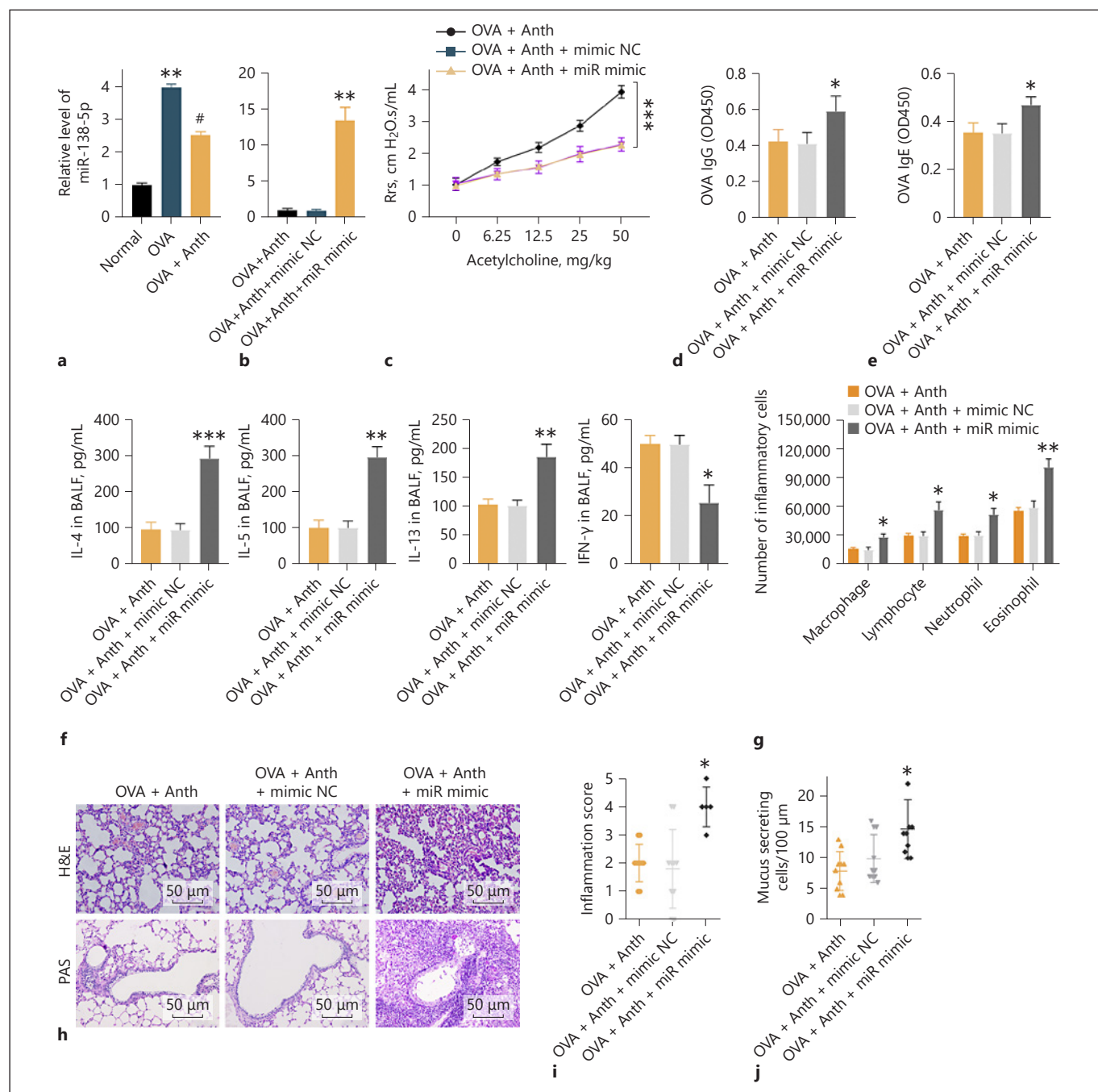


Fig. 2. Anthocyanins downregulated the level of miR-138-5p in asthmatic mouse models. The asthmatic model mice treated with anthocyanins were infected with miR-138-5p. The expression of miR-138-5p in vivo was detected by RT-qPCR (**a**); transfection efficiency of the miR-138-5p mimic was detected by RT-qPCR (**b**); airway response ability in mice was measured by intravenously injecting 0, 6.25, 12.5, 25, and 50 μg/kg Ach (**c**); serum OVA-specific IgE in the serum and IgG levels in BALF were detected using ELISA kits (**d**, **e**); levels of IL-4, IL-5, IL-13 and IFN-γ in BALF were detected using ELISA kits (**f**); number of eosinophils, macrophages, lymphocytes, and neutrophils in BALF was measured using

a hemocytometer (**g**); HE staining and PAS staining were performed in the mouse lung tissues (**h**); lung inflammation scoring and mucus secretory cell counting (**i**, **j**). One-way ANOVA was used for data analysis. Tukey's multiple comparisons test was used for the post hoc test; compared with the normal group, ** $p < 0.01$; compared with the OVA group, # $p < 0.05$; compared with the OVA + Anth + mimic NC group, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Ach, acetylcholine; HE, hematoxylin and eosin; RT-qPCR, reverse-transcription quantitative polymerase chain reaction; OVA, ovalbumin; BALF, bronchoalveolar lavage fluid; ELISA, enzyme-linked immunosorbent assay; PAS, periodic acid-Schiff.

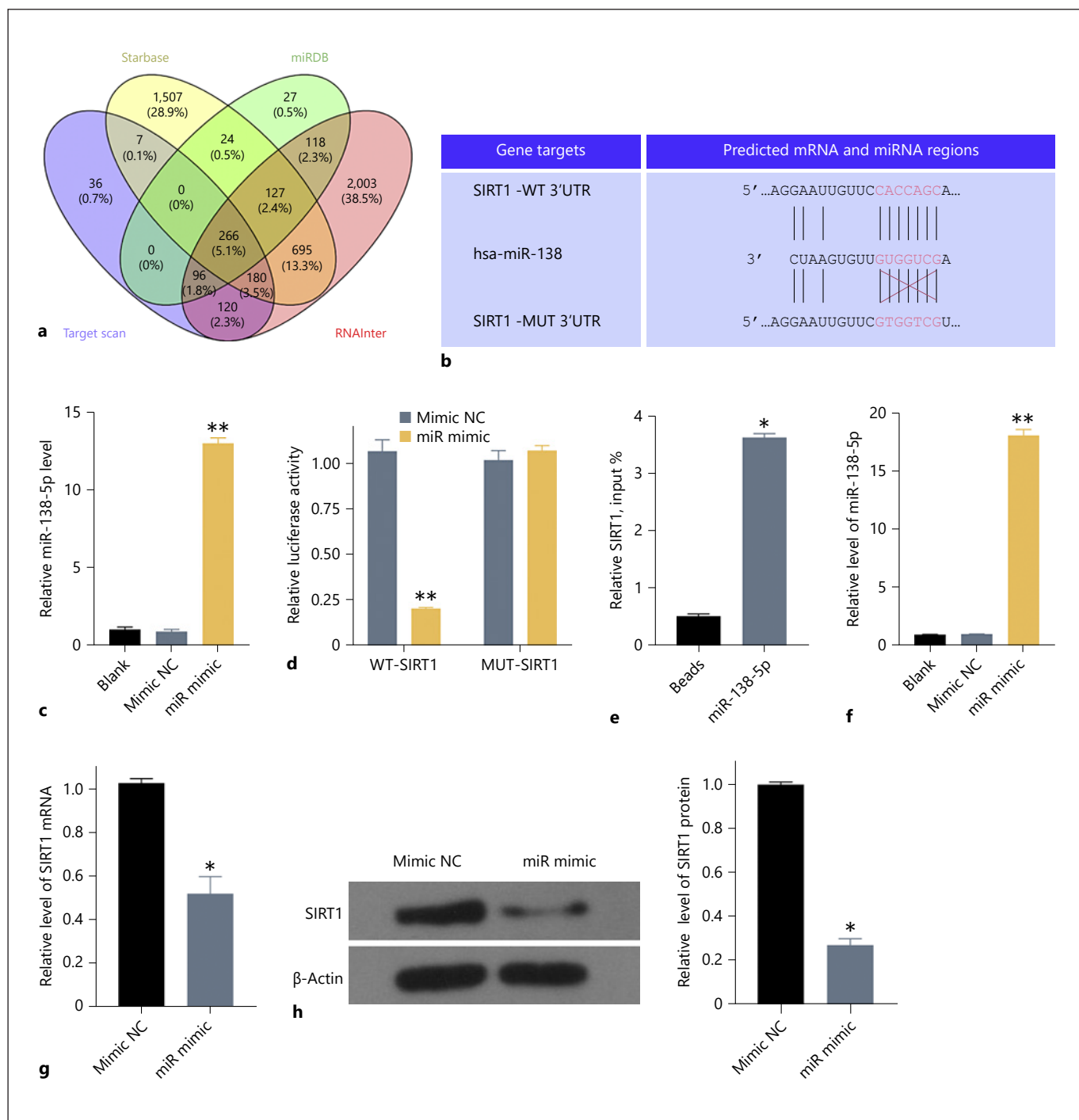


Fig. 3. miR-138-5p targeted SIRT1. The downstream target genes of miR-138-5p were analyzed and predicted using Starbase, miRDB, RNAInter, and TargetScan, and the intersection was obtained (a); TargetScan predicted that SIRT1 contained miR-138-5p-binding sites and mutation sites in its 3'-UTR (b); transfection efficiency of HEK293T cells transfected with the miR-138-5p mimic was detected by RT-qPCR (c); binding relationship of miR-138-5p and SIRT1 was verified by dual-luciferase and RNA pull-down assays (d, e); transfection efficiency of 16HBE14_o-160 trans-

fectected with miR-138-5p mimic was detected by RT-qPCR (f); mRNA and protein levels of SIRT1 were detected by RT-qPCR and WB (g, h). The experiment was repeated 3 times. One-way ANOVA was used for data analysis. Tukey's multiple comparisons test was used for the post hoc test. *Compared with the mimic NC or the beads group, $p < 0.05$; **compared with the mimic NC group, $p < 0.01$. SIRT, sirtuin; 3'-UTR, 3'-untranslated region; RT-qPCR, reverse-transcription quantitative polymerase chain reaction; WB, Western blot; HBE, human bronchial epithelial.

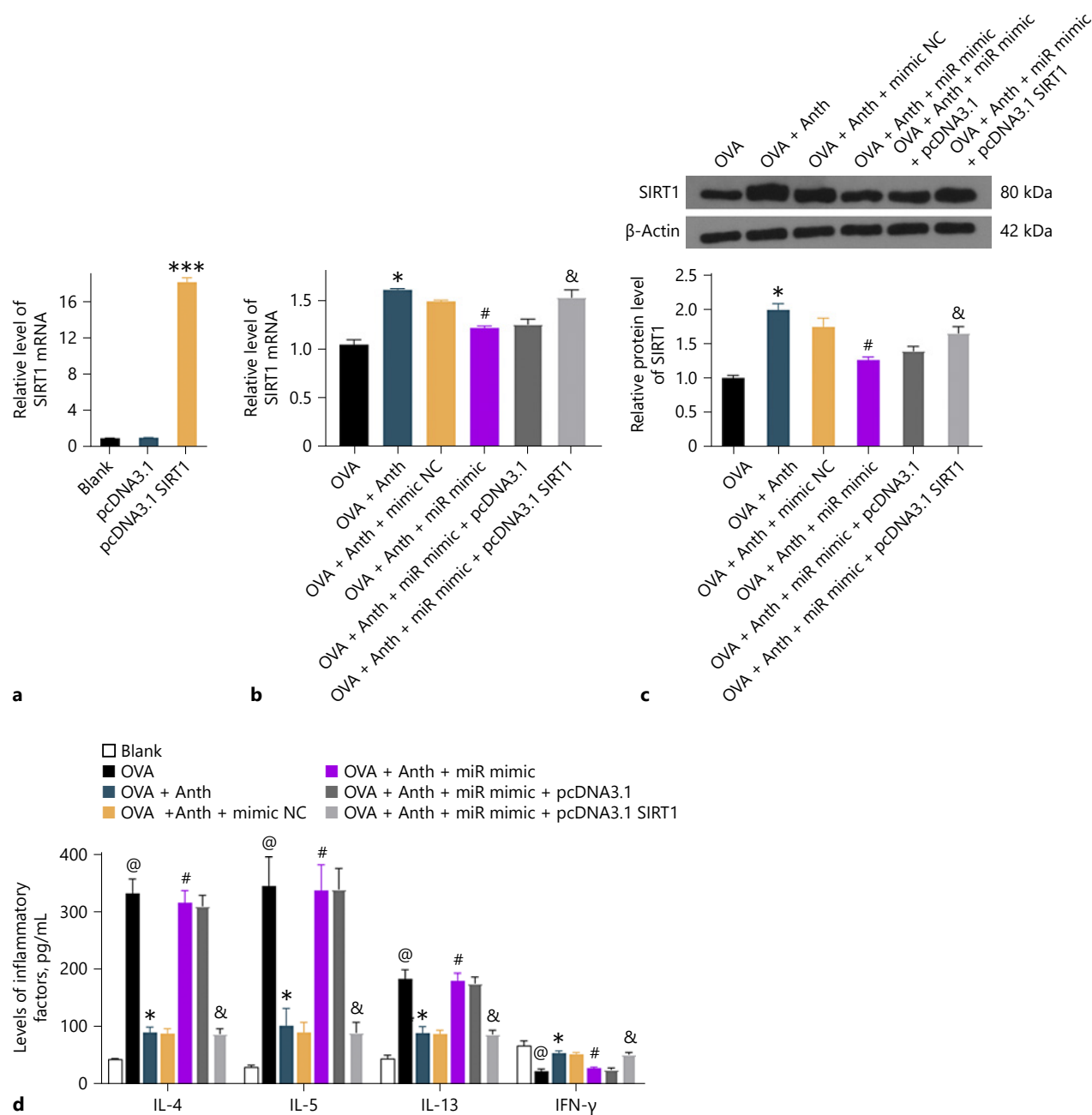


Fig. 4. Anthocyanins inhibited the release of inflammatory factors by downregulating the miR-138-5p/SIRT1 axis. The transfection efficiency of 16HBE14_o-160 transfected with pcDNA3.1-SIRT1 was detected by RT-qPCR (**a**); mRNA level of SIRT1 in 16HBE14_o-160 cells was detected by RT-qPCR (**b**); protein levels of SIRT1 were detected by WB (**c**); levels of inflammatory factors released by 16HBE14_o-160 cells were detected using ELISA kits (**d**). The cell experiment was repeated 3 times. One-way ANOVA was used for data analysis. Tukey's multiple comparisons test was used for the

post hoc test. Compared with the pcDNA3.1 group, *** $p < 0.001$; compared with the OVA group, * $p < 0.05$; compared with the OVA + Anth group, # $p < 0.05$; compared with the OVA + Anth + mimic NC group, & $p < 0.05$; compared with the OVA + Anth + miR + pcDNA3.1 group, & $p < 0.05$; compared with the blank group, @ $p < 0.05$. SIRT, sirtuin; HBE, human bronchial epithelial; RT-qPCR, reverse-transcription quantitative polymerase chain reaction; WB, Western blot; ELISA, enzyme-linked immunosorbent assay; OVA, ovalbumin; Anth, anthocyanin.

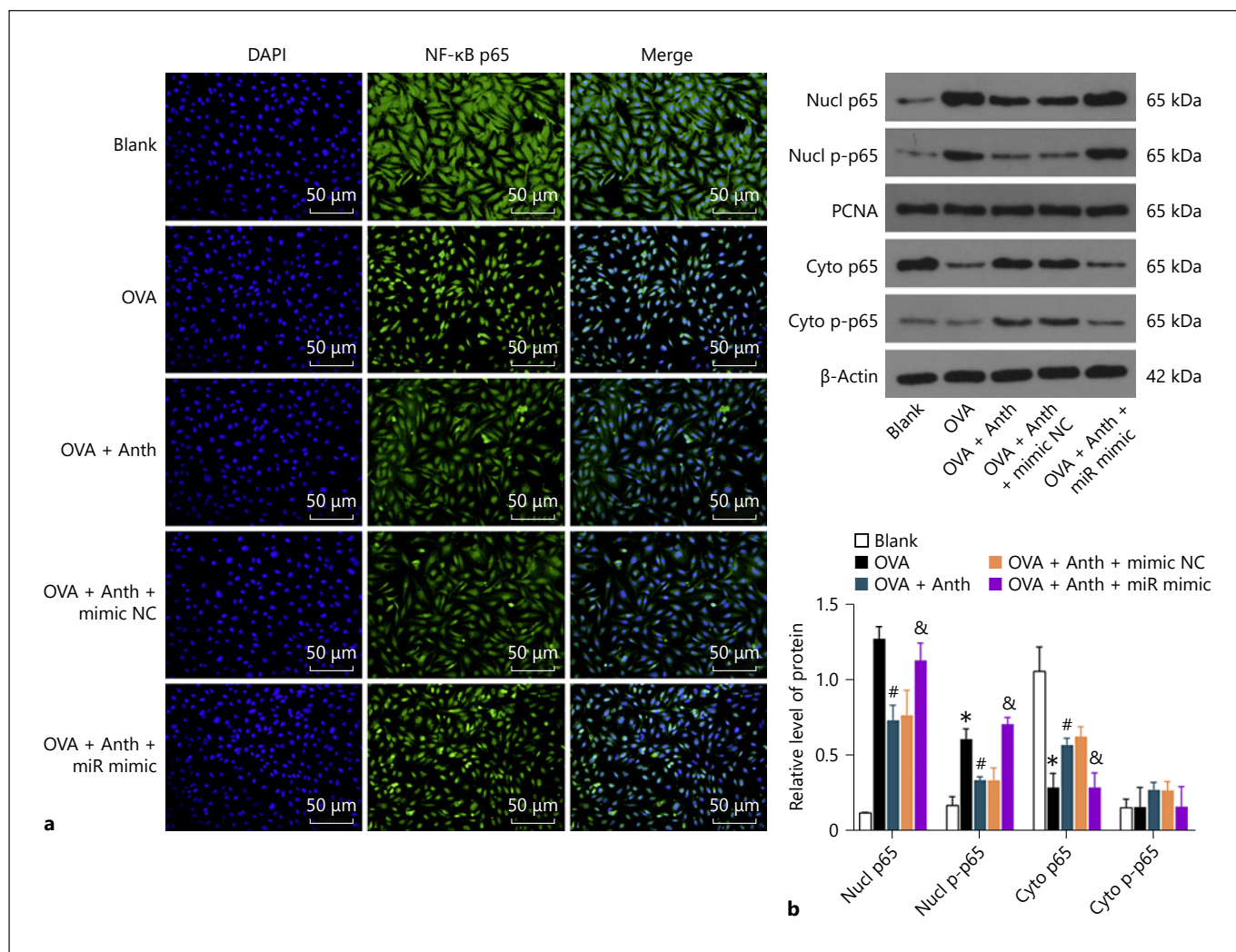


Fig. 5. Anthocyanins inhibited nuclear translocation of NF-κB by mediating the miR-138-5p/SIRT1 axis. The NF-κB p65 nuclear translocation in 16HBE140-160 cells was detected by immunofluorescence (a); protein levels of p-p65 and p65 in the nucleus and the cytoplasm were detected by WB (b). The cell experiment was repeated 3 times. One-way ANOVA was used for data analysis.

Tukey's multiple comparisons test was used for the post hoc test. Compared with the blank group, * $p < 0.05$; compared with the OVA group, # $p < 0.05$; Compared with the OVA + Anth + miR mimic + pcDNA3.1 group, & $p < 0.05$. NF-κB, nuclear factor-κB; SIRT, sirtuin; HBE, human bronchial epithelial; WB, Western blot; OVA, ovalbumin; Anth, anthocyanin.

all $p < 0.05$). Hematoxylin and eosin and PAS staining demonstrated that the inflammatory infiltration and mucus secretion were aggravated (Fig. 2h–j, all $p < 0.05$). Altogether, anthocyanins alleviated airway inflammation in mice with asthma by inhibiting miR-138-5p.

miR-138-5p Targeted SIRT1

Subsequently, the downstream target genes were analyzed and predicted using Starbase, miRDB, RNAinter, and TargetScan, and the intersection was obtained (Fig. 3a). Among them, we focused on SIRT1. Studies

have revealed that the SIRT1-related pathway is closely related to airway inflammation in asthma, and SIRT1 can regulate some transcription factors and inhibit the release of inflammatory factors [14, 25]. Then, TargetScan predicted that SIRT1 included miR-138-5p-binding sites in its 3'-UTR (Fig. 3b). Whether miR-138-5p could bind to SIRT1 was verified by the dual-luciferase assay. After HEK293T cells were cotransfected with the miR-138-5p mimic (the transfection efficiency was manifested in Fig. 3c), the fluorescence in the SIRT1-WT 3'-UTR group was significantly decreased ($p < 0.01$), while there was no

significant difference in the SIRT1-MUT 3'-UTR group (Fig. 3d). Subsequently, 16HBE14_o-160 cells were transfected with biotinylated miR-138-5p for RNA pull-down analysis, and the interaction between miR-138-5p and SIRT1 was verified (Fig. 3e). After the miR-138-5p mimic was delivered into 16HBE14_o-160 cells (the transfection efficiency was shown in Fig. 3f), RT-qPCR and Western blot (WB) experiments elicited that the SIRT1 levels were significantly decreased (Fig. 3g, h, all $p < 0.05$). The results above suggested that miR-138-5p targeted SIRT1 and negatively regulated its expression.

Anthocyanins Inhibited the Release of Inflammatory Factors by Downregulating the miR-138-5p/SIRT1 Axis

The 16HBE14_o-160 cells were treated with OVA to establish in vitro models. ELISA results showed that IL-4, IL-5, and IL-13 were increased, and the level of IFN- γ was decreased after OVA treatment, while anthocyanin intervention could significantly decrease OVA-induced increases of the protein levels of IL-4, IL-5, and IL-13 and increase the level of IFN- γ (Fig. 4d, all $p < 0.05$). The 16HBE14_o-160 cells were transfected with the miR-138-5p mimic or pcDNA3.1-SIRT1 and its corresponding control (the transfection efficiency was presented in Fig. 4a). RT-qPCR and WB results demonstrated that compared with the OVA group, SIRT1 was significantly upregulated in the OVA + Anth group, while compared with the OVA + Anth + mimic NC group, SIRT1 in the OVA + Anth + miR mimic group was decreased, and compared with the OVA + Anth + miR mimic + pcDNA3.1 group, SIRT1 was upregulated in the OVA + Anth + miR mimic + pcDNA3.1-SIRT1 group (Fig. 4b, c, $p < 0.05$). ELISA results showed that the overexpression of miR-138-5p partially reversed the therapeutic effect of anthocyanins, while the overexpression of SIRT1 antagonized the effect of miR-138-5p overexpression (Fig. 4d, all $p < 0.05$). Briefly, anthocyanins downregulated miR-138-5p to upregulate SIRT1 and inhibited the release of inflammatory factors.

Anthocyanins Downregulated NF- κ B by Mediating the miR-138-5p/SIRT1 Axis, Thus Alleviating Airway Inflammation in Asthmatic Mice

Activation of the NF- κ B p65 pathway and nuclear translocation are closely related to the development of asthma inflammation [26]. The effects of anthocyanins and miR-138-5p on regulating NF- κ B were verified by immunofluorescence. OVA induction could cause nuclear translocation of NF- κ B p65, while after anthocyanin intervention, the nuclear translocation of NF- κ B p65 was

significantly decreased, and the overexpression of miR-138-5p could weaken the effect of anthocyanins (Fig. 5a). Similarly, the NF- κ B p65 pathway in 16HBE14_o-160 cells was analyzed by WB. Compared with the OVA group, the levels of p-p65 and p65 in the nucleus were significantly decreased, the levels of p-p65 and p65 in the cytoplasm were increased in the OVA + Anth group, while in the OVA + Anth + miR-138-5p mimic group, the levels of p-p65 and p65 in the nucleus were increased, and the levels of p-p65 and p65 in the cytoplasm were decreased compared with the OVA + Anth + mimic NC group (Fig. 5b, all $p < 0.05$). The results above suggested that anthocyanins inhibited the NF- κ B activation via the miR-138-5p/SIRT1 axis, thus inhibiting airway inflammation in asthmatic mice.

Discussion

Asthma is a common disease of the respiratory system with increasing incidence and prevalence worldwide [4]. A previous study has demonstrated that anthocyanins are important in asthma treatment [19]. Our study found that anthocyanins inhibited airway inflammation by blocking the NF- κ B pathway via the miR-138-5p/SIRT1 axis in asthmatic mice.

The protective effect of anthocyanins on the pathogenesis of various inflammatory diseases has been reported, especially in asthma [24]. We established asthmatic mouse models by OVA sensitization. The airway resistance is significantly increased in asthma [27]. Our results demonstrated that anthocyanins significantly reduced the OVA-induced airway resistance. The airway inflammation is characterized by an elevated IgE expression in the serum [28]. IL-4, IL-5, IL-13, and eosinophil counting are elevated in asthma [29]. Allergic asthma is characterized by airway smooth-muscle layer thickening [30]. Our results found that anthocyanins inhibited AHR and decreased serum OVA-specific IgE level, inflammatory cells, inflammatory factors, and IgG levels in BALF in asthmatic mice. The inflammatory infiltration was clearly reduced, airway structure was relatively complete, and mucus secretion and the proliferation of goblet cells were alleviated in lung tissue of asthmatic mice after anthocyanin intervention. It is consistent with that anthocyanins alleviate OVA-induced inflammation and eosinophil increase in allergic asthmatic mouse models [9]. Collectively, anthocyanins inhibited AHR and airway inflammation in asthmatic mice.

miR-138 is involved in the inflammatory response in asthma [13]. We speculated that anthocyanins might

play a role in OVA-induced asthma through regulating miR-138-5p. Our results demonstrated that miR-138-5p expression was significantly upregulated in OVA-induced asthma mouse model, while downregulated after anthocyanin intervention. Consistently, miR-138 is upregulated in asthma [31]. To further ascertain the role of anthocyanins in asthma, we transfected the miR-138-5p mimic into mice. Airway resistance was increased; the levels of OVA-specific IgE in serum and IgG in BALF were increased; the levels of IL-4, IL-5 and IL-6 were increased, the levels of IFN- γ were decreased; inflammatory cells in BALF were increased; and the inflammatory infiltration and mucus secretion were aggravated. Inhibition of miR-138-5p led to attenuated inflammatory responses upon TNF- α and IL-6 stimulation, alleviated allergic symptoms in mice, and reduced OVA-specific IgG release [32]. In conclusion, anthocyanins alleviated airway inflammation in mice with asthma by inhibiting miR-138-5p.

The downstream targets of miR-138-5 were further explored. Starbase, miRDB, RNAinter, and TargetScan were used to predict the downstream binding sites of miR-138-5. SIRT1 was obtained. The binding relationship of miR-138-5 and SIRT1 was verified by dual-luciferase and RNA pull-down assays. After the miR-138-5p mimic was delivered into 16HBE14 $_0$ -160 cells, SIRT1 levels were significantly decreased. Consistently, miR-138-5p targets SIRT1, thus inhibiting inflammatory responses in acute lung injury [33]. Briefly, miR-138-5p targeted SIRT1 and negatively regulated its expression. Subsequently, 16HBE14 $_0$ -160 cells were cotransfected with miR-138-5p mimic and pcDNA3.1-SIRT1. SIRT1 expression was significantly upregulated after anthocyanin treatment, while decreased after transfection with miR-138-5p mimic and upregulated after cotransfection with the miR-138-5p mimic and pcDNA3.1-SIRT1. Furthermore, overexpression of miR-138-5p partially reversed the therapeutic effect of anthocyanins, while the overexpression of SIRT1 antagonized the effect of miR-138-5p overexpression. Consistently, SIRT1 regulates the level of IL-6, thus affecting the pulmonary function of asthma patients [34]. SIRT1 activation decreases IL-4, IL-5, and IL-13 levels in allergic rhinitis [35]. In conclusion, anthocyanins downregulated miR-138-5p expression to upregulate SIRT1 expression, thus inhibiting the release of inflammatory factors.

The NF- κ B p65 pathway activation and nuclear translocation are closely associated with asthma inflammation development [26]. Our results showed that OVA induction caused nuclear translocation of NF- κ B p65, while af-

ter anthocyanin intervention, the nuclear translocation of NF- κ B p65 was significantly decreased, and the overexpression of miR-138-5p weakened the effect of anthocyanins. Similarly, in 16HBE14 $_0$ -160 cells, the levels of p-p65 and p65 in nucleus were significantly decreased, the levels of p-p65 and p65 in cytoplasm were increased after anthocyanin intervention, while after transfection with miR-138-5p mimic, the levels of p-p65 and p65 in nucleus were increased, and the levels of p-p65 and p65 in cytoplasm were decreased. It is consistent with that anthocyanins play the role of anti-inflammatory by modulating the NF- κ B pathway [17]. miR-138-5p can directly target the NF- κ B p65 transcript [36]. Collectively, anthocyanins inhibited the activation of the NF- κ B pathway via the miR-138-5p/SIRT1 axis, thus inhibiting airway inflammation in asthmatic mice.

In summary, this study supported that anthocyanins inhibited airway inflammation by blocking the NF- κ B pathway via the miR-138-5p/SIRT1 axis in asthmatic mice. These results are helpful to provide new ideas and new directions for clinical treatment of asthma. However, the regulatory mechanism of anthocyanins on miR-138-5p has not been deeply studied. Further study is needed to explore the regulatory mechanism of anthocyanins on miR-138-5p from the perspective of epigenetics.

Statement of Ethics

This study was performed following the approval from the Ethical Committee of Civil Aviation General Hospital. All the animal experiments were implemented on the guide for the care and use of laboratory animals.

Conflict of Interest Statement

The authors declare that they have no competing interests.

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Author Contributions

Y.L. contributed to the study concepts, study design, and guarantor of integrity of the entire study; M.Q.Z. and Z.W.H. contributed to the definition of intellectual content; L.L. contributed to the literature re-

search; Y.L. and M.Q.Z. contributed to manuscript preparation; X.S.Q. and L.L. contributed to manuscript editing and review; H.J.Z., Y.L., and Z.W.H. contributed to the experimental studies and data acquisition; X.S.Q. and Y.L. contributed to data analysis and statistical analysis. All the authors read and approved the final manuscript.

Data Availability Statement

All the data generated or analyzed during this study are included in this published article.

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