

RESEARCH ARTICLE

Chrysophanol attenuates airway inflammation and remodeling through nuclear factor-kappa B signaling pathway in asthma

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Chrysophanol (CHR), a purified active constituent extracted from *Rheum palmatum* L., possesses anti-inflammatory activity. This study aimed to evaluate its effects on asthma-associated airway inflammation and remodeling. BALB/c mice were sensitized and challenged by ovalbumin (OVA) and administrated with different doses of CHR. We found that CHR decreased OVA-induced pulmonary inflammation: the levels of interleukin (IL)-4, IL-5, and IL-13, tumor necrosis factor (TNF)- α , and inducible nitric oxide synthase were downregulated. CHR also attenuated airway remodeling induced by OVA challenge—CHR inhibited pulmonary α -smooth muscle actin expression. Moreover, both the nuclear translocation and activity of NF- κ B p65 were inhibited by CHR in the asthmatic lung. Enhanced autophagy was initiated in the lung by OVA challenge as evidenced by upregulated light chain 3 beta, autophagy-related protein 5, and Beclin 1. CHR suppressed OVA-induced alterations in these autophagy-related molecules. In vitro, CHR (2 or 20 μ M) was used to treat human pulmonary epithelial BEAS-2B cells in the presence of 10 ng/ml recombinant TNF- α . CHR not only exhibited the antiproliferation effect but also inhibited the activation of nuclear factor-kappa B (NF- κ B) signaling pathway in TNF- α -treated BEAS-2B cells. In conclusion, our study indicates that CHR has the potential to ameliorate asthma.

KEYWORDS

airway remodeling, asthma, autophagy, chrysophanol, inflammation, NF- κ B pathway

1 | INTRODUCTION

Asthma is one of the most common chronic, noncommunicable syndromes in both children and adults, and about 334 million people suffer from this disease worldwide (Papi, Brightling, Pedersen, & Reddel, 2018; Vos et al., 2012). Treatment cost of this disease leads to an escalating economic burden (Loftus & Wise, 2015). Asthma is a heterogeneous condition characterized by variable respiratory symptoms and airflow limitation (O'Byrne, Naji, & Gauvreau, 2012), one of them is airway inflammation. The infiltration of inflammatory cells, such as lymphocytes, activated mast cells, and type 2 T cell helper cells (Th2), has been recognized to cause asthmatic airway inflammation. The main

Th2 cytokines—interleukin (IL)-4, IL-5, and IL-13—are pivotal proinflammatory cytokines in asthma that contribute to airway inflammation and remodeling (Richter et al., 2001; Wynn, 2004).

Airway remodeling in asthma involves various pathophysiological characteristics, including increased smooth muscle mass, vascular changes, subepithelial fibrosis, and particular increased numbers of activated fibroblasts/myofibroblasts (can be marked by α -smooth muscle actin [α -SMA]; Al-Muhsen, Johnson, & Hamid, 2011; Tagaya & Tamaoki, 2007). The increased airway smooth muscle mass will cause airway narrowing, thus promoting dyspnea in patients with asthma (Al-Muhsen et al., 2011; Prakash, 2013). Therefore, it is likely that decreasing the excessive airway smooth muscle cells may

attenuate some of the symptoms in asthma. Although increasing advances in understanding the mechanisms of asthma have been made, effective therapies controlling the refractory inflammation and airway remodeling in asthma are still limited.

During the past few decades, a number of natural herbs have been used for the prevention and treatment of asthma (Bao et al., 2017; Cho et al., 2015; Chunlian et al., 2014). Chrysophanol (CHR), a member of the anthraquinone family, was originally extracted from *Rheum palmatum* L., and it has been shown to display multiple pharmacological effects, such as anti-inflammation (S. J. Kim et al., 2010), antitumor (Ni et al., 2012), antiviral (Chang, Huang, Lin, Tsou, & Lin, 2014), and antiproliferation (Hu, Zhang, Meng, Wang, & Wang, 2014). Specifically, the number of studies focusing on CHR protective effects against lung diseases has risen (Li et al., 2016; Ni et al., 2014; C. Zhang et al., 2017). Li et al. reported that CHR attenuated paraquat-induced lung injury through inhibiting proinflammatory NF- κ B pathway (Li et al., 2016). However, the precise cellular mechanisms underlying the effects of CHR on asthma have yet to be revealed.

Autophagy plays an essential role in pulmonary inflammation during asthma progress (Gu et al., 2017; J. N. Liu et al., 2016). Airway inflammation involves the induction of autophagy (Yin et al., 2017). Blockade of autophagy was demonstrated to attenuate asthmatic inflammation and airway remodeling (T. Liu et al., 2017). Antiasthma effects of CHR have been reported by C. Zhang et al. (2017). Nonetheless, whether such protective effects of CHR are associated with autophagy regulation is unknown.

In this study, ovalbumin (OVA)-challenged asthmatic mice and tumor necrosis factor (TNF)- α -stimulated human pulmonary epithelial BEAS-2B cells were used as the in vivo and in vitro models, respectively. The effects of CHR against airway inflammation and remodeling and the underlying mechanisms were investigated.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

All animal experiments were conducted with the approval from the Medical Ethics Committee of The First Affiliated Hospital of Henan University of Traditional Chinese Medicine (Approval number: DWLL16010004), and all procedures conformed to the Guide for the Care and Use of Laboratory Animals. Male BALB/c mice (20–25 g) aged 8–10 weeks were purchased from Liaoning Changsheng Bio. (Benxi, Liaoning, China). These animals were acclimatized for a week, fed with a regular diet, and housed in a specific pathogen-free state under a strict light cycle (12:12 hr) at a temperature of $22 \pm 1^\circ\text{C}$ and a relative humidity of $50 \pm 5\%$.

2.2 | Preparation of CHR

CHR with a purity >98% was purchased from Abcam (Ab142268; Cambridge, MA, USA) and dissolved in DMSO, and the storage concentration for CHR was 2 mg/ml. Then, the CHR was diluted with

sterilized physiological saline (1% DMSO and 1% Tween 80; N. Zhang et al., 2014).

2.3 | OVA-induced asthma model and experimental grouping

Mice were sensitized with 20- μg OVA (Sigma Aldrich, St. Louis, MO, USA) in normal saline by intraperitoneal (i.p.) injection at Days 0, 14, and 28. From Days 21 to 42, mice were challenged with aerosolized 1% OVA for 30 min three times a week. The nonsensitized mice were treated with physiological saline (containing 1% DMSO and 1% Tween 80) in the same way (Gong et al., 2014; Kistemaker et al., 2014).

For the experiment, mice were divided into ($n = 12$ per group): (1) Control (Con) group: mice were treated with sterilized physiological saline (containing 1% DMSO and 1% Tween 80); (2) OVA group: asthmatic mice that were given CHR vehicle; (3–5) OVA + CHR (L, M, or H) group: asthmatic mice that were treated with 0.1, 1, or 10 mg/kg CHR i.p. once a day from Day 35 to Day 39. Furthermore, (6) OVE + dexamethasone (DEX) group ($n = 6$): asthmatic mice that were treated with 1.0 mg/kg DEX (Ruichengkelong, Shanxi, China) i.p. 1 hr before aerosolization with OVA. Finally, mice were sacrificed at Day 43 (24 hr after the last OVA treatment).

2.4 | Cell counts in bronchoalveolar lavage fluid

Mouse lungs were lavaged with 0.5-ml cold sterilized physiological saline for three times, and bronchoalveolar lavage fluid (BALF) was collected via a tracheal cannula. A total volume of approximately 1.5-ml BALF was instilled up and centrifuged at 1,500 rpm for 10 min at 4°C to separate cell pellet and supernatants. The total number of cells was counted using a cell counter and was recorded as the total number of inflammatory cells per ml. The collected cell pellet was smeared onto slides for cell classification and counting. Cells were stained with Wright-Giemsa (Nanjing Jiangcheng Bioengineering Institute, Nanjing, China), after which the number of leukocytes and eosinophils were recorded by counting under light microscopy (Olympus, Tokyo, Japan).

2.5 | Histological examination

The lung tissues were fixed with 4% paraformaldehyde for 30 min, embedded in paraffin wax, and then sectioned into 5- μm slices. For hematoxylin and eosin staining, the sections were stained with hematoxylin (Solarbio Science & Technology, Co., Ltd, Beijing, China) and eosin (Sangon Bio., Shanghai, China; C. Zhang et al., 2017). For Masson's staining, the sections were stained with Regaud hematoxylin stain (Solarbio Science & Technology Co., Ltd), ponceau acid-fuchsin stain (Sinopharm Chemical Reagent, Shanghai, China), and aniline blue stain (C. Zhang et al., 2017). Morphological features of the sections were observed under a light microscope (Olympus). All images were taken at $200\times$ magnification.

2.6 | Enzyme-linked immunosorbent assay

Serum samples from mice were collected at the end of animal experiments. The serum levels of immunoglobulin E (IgE) were detected by Mouse IgE enzyme-linked immunosorbent assay (ELISA) kit (EK2751, MultiSciences, Hangzhou, Zhejiang, China). The concentrations of IL-4, IL-5, IL-13, and TNF- α in the lung tissues were determined using Mouse IL-4, IL-5, IL-13, and TNF- α ELISA kits (EK2041/2, EK2051, EK2131/2, and EK282/3-96; MultiSciences) according to the manufacturer's protocols.

2.7 | Quantitative real-time polymerase chain reaction

Total RNAs were extracted from the lung tissues, and the concentration was detected by an ultraviolet spectrophotometer (NANO 2000, ThermoFisher, Waltham, MA, USA). Complementary DNAs were synthesized from RNA templates via Super M-MLV reverse transcriptase (BioTeke, Beijing, China). The complementary DNAs were then amplified using SYBR Green (Solarbio Science & Technology, Co., Ltd) and 2 $\times$ Power Taq polymerase chain reaction (PCR) MasterMix (BioTeke). Primers were shown in Table 1. The reaction condition of reverse transcription was as follows: 25°C for 10 min, 42°C for 50 min, and 80°C 10 min. The PCR amplification conditions were as follows: 94°C for 5 min, 40 cycles of 94°C for 10 s, 60°C for 20 s, and 72°C for 30 s and then 72°C for 2.5 min, 40°C for 1.5 min, melting from 60°C to 94°C (every 1°C for 1 s), and finally, 25°C for 1–2 min. PCR reaction was performed on Exicycler™ 96 Real-Time Quantitative Thermal Block (Bioneer, Daejeon, Korea). Data were determined via the $2^{-\Delta\Delta CT}$ method.

2.8 | Western blot analysis

The tissue and cell samples were collected and lysed with RIPA Lysis buffer (Beyotime, Beijing, China) containing 1% phenylmethylsulphonyl

fluoride (Beyotime). The protein concentration was measured by a BCA Protein Assay Kit (Beyotime). Primary antibodies for anti- α -SMA (1:1,000; Proteintech, Rosemont, IL, USA), anti-inducible nitric oxide synthase (iNOS, 1:500; Proteintech), antimicrotubule-associated protein 1 light chain 3 beta (LC3B, 1:500; Sangon Bio.), antiautophagy-related protein 5 (Atg 5; 1:500; Sangon Bio.), anti-Beclin 1 (1:500; Proteintech), anti-IkB α (1:500; Proteintech), anti-p-IkB α (1:500; CST, Danvers, MA, USA), anti-NF- κ B p65 (1:500; CST), anti-p-ERK (1:1,000; CST), anti-ERK (1:1,000; CST), anti-p-p38 (1:400; Bioss, Beijing, China), anti-p38 (1:400; Bioss), anti-Histone H3 (1:500; Proteintech), and β -actin (1:500; Bioss) were used in this study. Equal amounts of protein samples were separated by 10–15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA). Then, the PVDF membranes were blotted with the appropriate primary antibody at 4°C overnight, followed by incubation with a horseradish peroxidase (HRP)-labeled Goat Anti-Rabbit IgG or HRP-labeled Goat Anti-Mouse IgG secondary antibody (1:5,000; Beyotime) for 1 hr at room temperature. The relative intensities of the protein bands were visualized by ECL detection reagent (Beyotime) and normalized to the corresponding β -actin. Quantitative analysis of the blots was performed by Gel-Pro-Analyzer software (Version 4.0, Media Cybernetics, Rockville, MD, USA), and the experiments have repeated a minimum of three times.

2.9 | Immunohistochemistry

Paraffin-embedded tissue sections (5- μ m thickness) were first incubated in xylene and then rehydrated in graded ethanol solutions. Then, the slides were blocked with goat serum (Solarbio Science & Technology, Co., Ltd) for 15 min at room temperature. The sections were immunostained with primary antibody anti- α -SMA (1:200, Proteintech) and anti-iNOS (1:100; Proteintech) overnight at 4°C. After washing the slides with phosphate buffer solution (PBS) for three times, the sections were incubated with secondary antibody at 37°C for 30 min. Sections were then washed with PBS three times and incubated in HRP-labeled Streptavidin (Beyotime) at 37°C for 30 min. Counterstaining was performed using hematoxylin (Solarbio Science & Technology, Co., Ltd), and the slides were visualized under a light microscope (Olympus); all images were taken at 400 $\times$ magnification.

2.10 | Immunofluorescence

Paraffin-embedded tissue sections (5- μ m thickness) were first incubated in xylene and then rehydrated in graded ethanol solutions. Tissue sections were retrieved in antigen retrieval solution for 10 min followed by a 15-min cooling at room temperature. Sections were blocked in goat serum (Solarbio Science & Technology, Co., Ltd) for 15 min and then incubated with anti- α -SMA (1:100; Proteintech) and anti-LC3B (1:50; Santa Cruz Biotechnology, Inc, Dallas, Texas, USA) antibody at 4°C overnight. Then, the slices

TABLE 1 Primer list for real-time PCR

Primer name	Sequence (5'–3')
iNOS F	TGGAGCGAGTTGTGGATTGTC
iNOS R	GTGAGGGCTTGGCTGAGTGA
LC3B F	TAACCAAGCCTTCTTCTCC
LC3B R	TTGCTGTCCCGAATGTCTCC
Atg 5 F	AAGGATGCGGTTGAGGCT
Atg 5 R	GCTCCGTCGTGGTCTGAT
Beclin 1 F	GCTGGAGTTGGATGACGAA
Beclin 1 R	TGATTGTGCCAACTGTCC
β -actin F	CTGTGCCCATCTACGAGGGCTAT
β -actin R	TTTGATGTCACGCACGATTTC

Abbreviations: Atg 5, autophagy-related protein 5; F, forward; iNOS, inducible nitric oxide synthase; LC3B, light chain 3 beta; PCR, polymerase chain reaction; R, reverse.

were incubated with the secondary antibodies FITC-labeled Goat Anti-Rabbit IgG (1:200; Beyotime) and Cy3-labeled Goat Anti-Mouse IgG (1:200; Beyotime) at room temperature for 90 min, respectively. Both antibodies were diluted in 1% goat serum albumin in PBS. Samples were stained with 4',6-diamidino-2-phenylindole (Beyotime) and observed in a fluorescence microscope (Olympus); all images were taken at 400 $\times$ magnification.

2.11 | Electrophoretic mobility shift assay

Nuclear proteins were extracted by Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime). The protein concentration was measured by a BCA Protein Assay Kit (Beyotime). Biotin-labeled NF- κ B was purchased from Beyotime (sequence: 5'-AGTTGAGGGGACTTCCCAGG C-3'). Nuclear proteins of each specimen were incubated with biotin-labeled probes for 20 min at room temperature. Protein-DNA complexes were separated from the free DNA probe by electrophoresis via a 6.5% native polyacrylamide gel. The separated proteins were transferred to PVDF membranes for 1 hr, cross-linked under the ultraviolet lamp for 10 min, and subjected to gentle shaking in blocking buffer containing streptavidin-HRP conjugate for 20 min. The membrane was washed and balanced, followed by addition of Lighten HRP-B Reagent A and Lighten HRP-B Reagent B. Proteins were finally analyzed with Gel-Pro-Analyzer software (Version 4.0, Media Cybernetics).

2.12 | Cell culture

Human pulmonary epithelial BEAS-2B cell line was obtained from Shanghai Zhong Qiao Xin Zhou Bio (Shanghai, China) and cultured in BEGM Medium (Lonza Clonetics, Basel, Switzerland) containing heat-inactivated fetal bovine serum (Hyclone Co., Logan, UT, USA), 100 units per ml of penicillin, and 100 mg/ml of streptomycin (Gibco®, Grand Island, NY, USA). The cells were kept at 37°C in a humidified atmosphere containing 5% CO₂.

2.13 | MTT assay

MTT assay was used to determine the cell viability in TNF- α -stimulated BEAS-2B cells in the presence or absence of CHR. Briefly, cells were seeded at the density of 4×10^3 per well in 96-well plates, treated with 10 ng/ml TNF- α and 2- or 20- μ M CHR for 12, 24, and 48 hr. Then, MTT was added and the plates were incubated for 4 hr at 37°C away from light. After removing the supernatant, DMSO was added into each well. The absorbance of each well was read on a microplate reader (BIOTEK, Winooski, VT, USA) at 570 nm. The optical density (OD) value was used for analysis.

2.14 | Statistical analysis

Data are presented as the mean $\pm$ standard deviation. Multiple comparisons were performed by one-way analysis of variance and Bonferroni's

post hoc test. Data analysis and plotting were conducted using GraphPad Prism 4.0 software (GraphPad Software, La Jolla, CA, USA); $p < .05$ was considered to indicate a statistically significant difference.

3 | RESULTS

3.1 | CHR attenuated the lung inflammation in OVA-challenged asthmatic mice in a dose-dependent manner

The effects of CHR on the accumulation of inflammatory cells including leukocytes and eosinophils in BALF and inflammatory infiltration in the lungs were examined after OVA challenge. As shown in Figure 1a, there was a significant increase in the number of total leukocytes in the BALF of OVA-challenged asthmatic mice as compared with that in Con group, whereas the number was significantly decreased in asthmatic mice treated with CHR in a dose-dependent manner ($p < .01$). The number of eosinophils in the BALF of CHR-treated asthmatic mice was also decreased significantly (Figure 1b, $p < .01$). Further, the ELISA assay showed that OVA challenge increased the serum concentration of IgE in mice, which was inhibited by CHR (Figure 1c, $p < .01$). Then, the production of IL-4, IL-5, IL-13, and TNF- α in the lung tissues was determined via ELISA analysis. As shown in Figure 1d, OVA markedly upregulated their generation in the mouse lung ($p < .01$ vs. Con), which was suppressed by CHR ($p < .05$ or $p < .01$). The anti-inflammatory effect of high dose CHR was comparable with that of DEX, a kind of corticosteroids that was usually used as an anti-inflammatory drug (Chauhan, Subhashini, Dash, & Singh, 2014; J. Kim, McKinley, Siddiqui, Bolgos, & Remick, 2004; Figure 1, $p < .01$). Similarly, hematoxylin and eosin staining indicated that the infiltration of inflammatory cells induced by OVA treatments was attenuated by CHR (Figure 1e). These results demonstrate that CHR inhibits the lung inflammation in OVA-challenged asthmatic mice in a dose-dependent manner.

3.2 | CHR downregulated iNOS and attenuated the airway remodeling in OVA-challenged asthmatic mice

It has been well documented that iNOS drives the production of NO that affects many features of asthma pathophysiology (Batra et al., 2007). The mRNA and protein levels of iNOS in the lung tissues were detected by quantitative real-time PCR and western blot analyses. We found a marked expression elevation of iNOS in asthmatic mice ($p < .01$ vs. Con; Figure 2a,b). CHR significantly abrogated iNOS upregulation in a dose-dependent manner. Immunohistochemistry staining of iNOS confirmed the findings from quantitative real-time PCR and western blot analysis (Figure 2c). Furthermore, to evaluate the extent of airway remodeling, the pulmonary expression levels of α -SMA were determined with western blot (Figure 2d) and immunohistochemistry (Figure 2e) analyses. Data showed that OVA challenge upregulated α -SMA expression, which was significantly reduced by CHR. Additionally, Masson's trichrome staining was performed to evaluate the extracellular matrix deposition in the lung tissues. Extensive

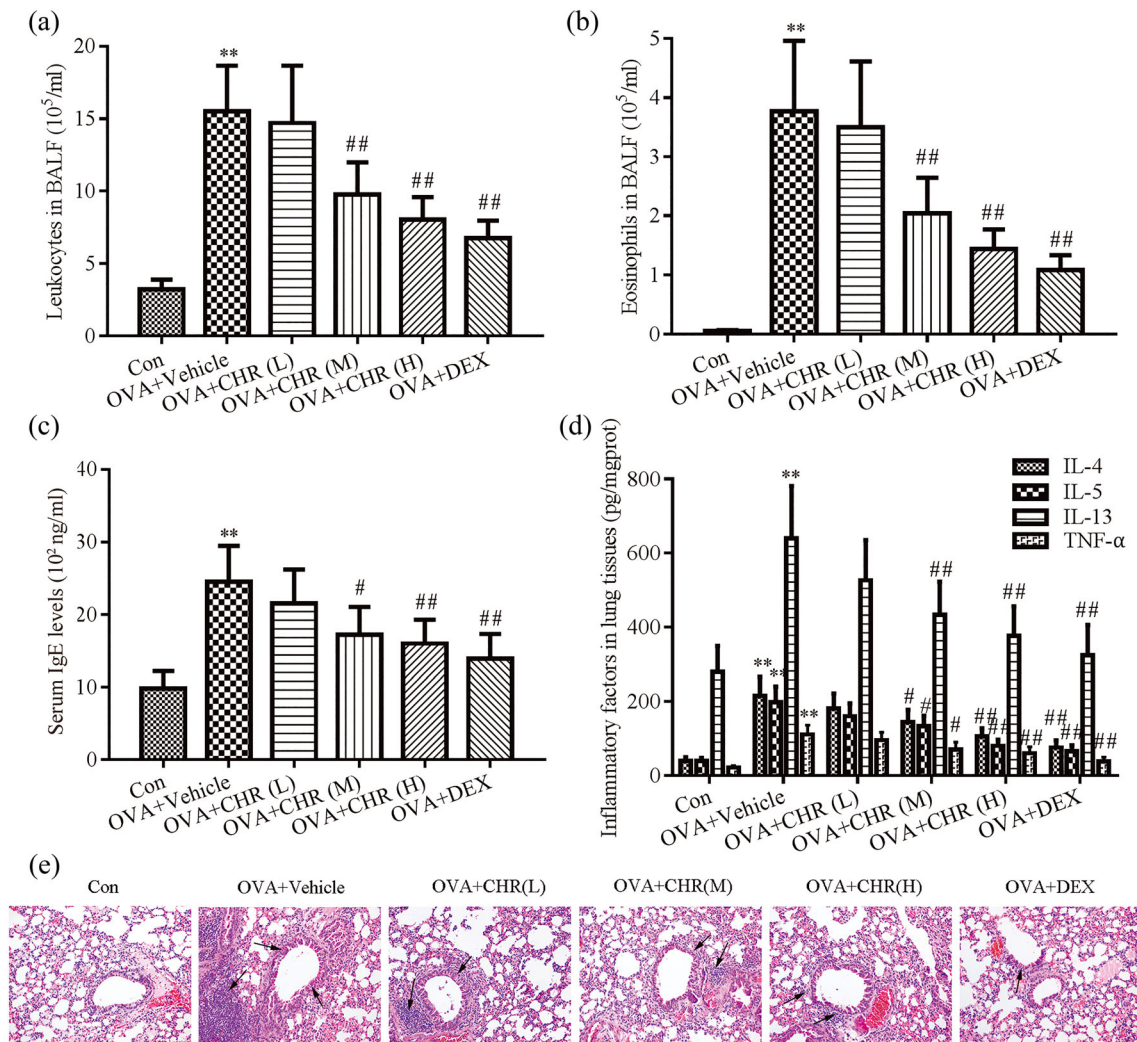


FIGURE 1 CHR attenuated the airway inflammation in OVA-challenged asthmatic mice in a dose-dependent manner. (a and b) Total numbers of leukocytes (a) and eosinophils (b) in the BALF of OVA-challenged asthmatic mice were counted after treatment with 0.1 mg/kg (L), 1.0 mg/kg (M), 10 mg/kg (H) CHR, or 1.0 mg/kg DEX. (c) The serum levels of OVA-IgE following treatment with or without OVA and/or CHR or DEX were evaluated by ELISA. (d) CHR inhibited the expression of IL4, IL-5, and IL-13 in the lung tissues after sensitization and challenged with OVA. Levels of IL4, IL-5, and IL-13 in the lung tissues were measured by ELISA. (e) Representative photomicrographs of H&E-stained lung sections. Arrows indicated infiltrated inflammatory cells. All images were at 200 $\times$ magnification. ** $p < .01$ versus Con group; ## $p < .01$ versus OVA + Vehicle group. BALF, bronchoalveolar lavage fluid; CHR, chrysophanol; DEX, dexamethasone; ELISA, enzyme-linked immunosorbent assay; H&E, hematoxylin and eosin; IgE, immunoglobulin E; IL, interleukin; OVA, ovalbumin [Colour figure can be viewed at wileyonlinelibrary.com]

depositions of blue-staining collagen and regional fibrotic foci were observed in the lung tissues of OVA-challenged mice, which were significantly reduced in CHR-treated asthmatic mice. OVA-challenged mice had marked fibrotic changes in alveolar areas, whereas CHR-treated asthmatic mice showed significantly less damage.

3.3 | CHR inhibited autophagy in the lung tissues of OVA-challenged asthmatic mice

To determine whether CHR regulates the pulmonary autophagy in OVA-challenged mice, we examined the expression of three autophagy-associated molecules, LC3B, Atg 5, and Beclin 1, with western blot analysis. Data showed that the relative mRNA and protein expression levels of LC3B were markedly increased in the OVA

group whereas were decreased in the OVA + CHR (M)/(H) group. Similar results were observed in Atg 5 and Beclin 1 ($p < .01$, Figure 3a,b). The expression of both fibroblast/myofibroblast marker α -SMA and autophagic marker LC3B was detected via immunofluorescent double staining analysis. We found that compared with the Con mice, the fluorescence of both α -SMA (green fluorescence) and LC3B (red fluorescence) was greatly enhanced by OVA, which was markedly reduced in the OVA + CHR (M)/(H) group (Figure 3c).

3.4 | CHR inhibited NF- κ B signaling pathway in OVA-challenged asthmatic mice

The protein levels of NF- κ B p65 and I κ B α were analyzed by western blot analysis. As shown in Figure 4a,b, OVA enhanced I κ B α

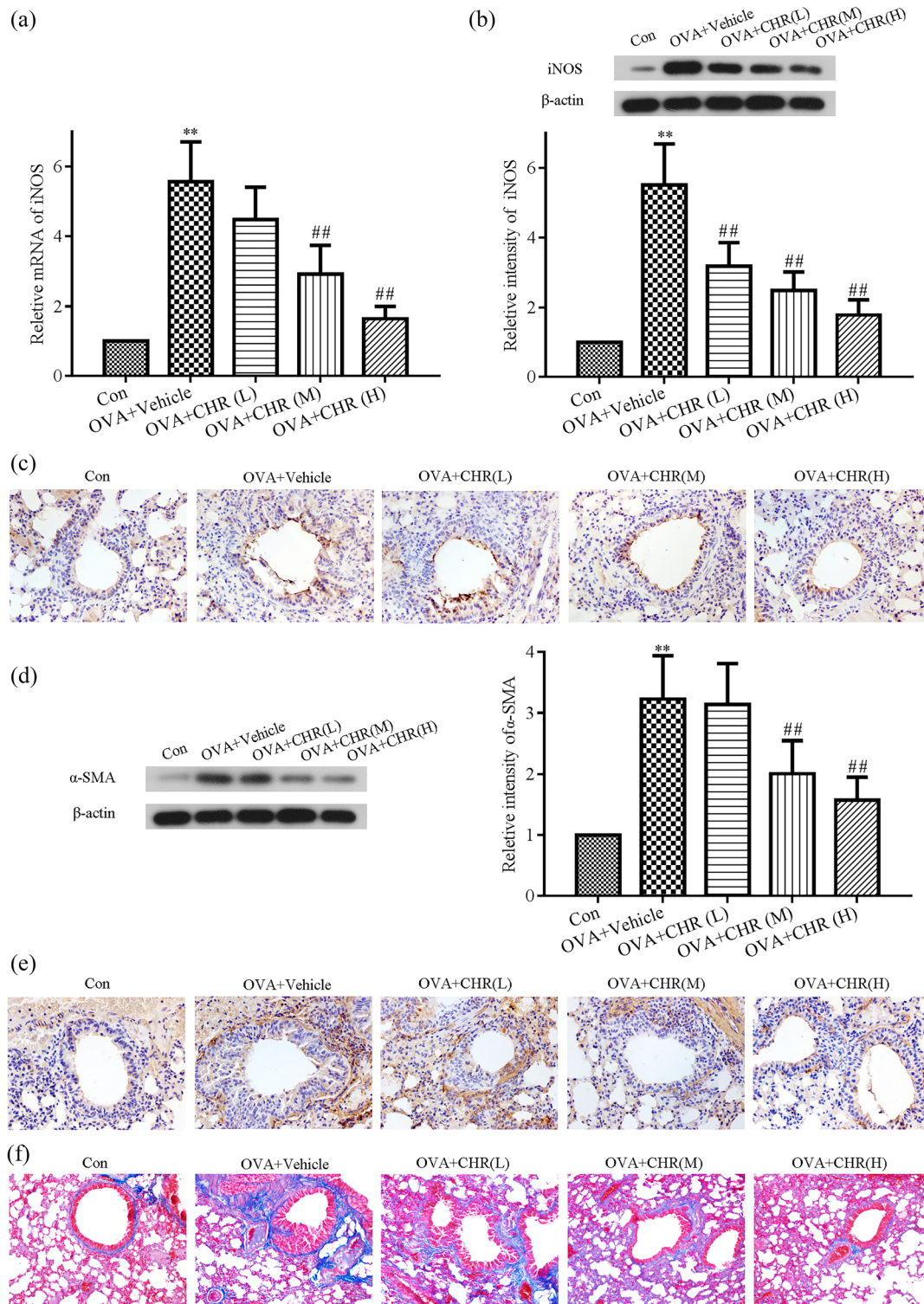


FIGURE 2 CHR attenuated the airway remodeling and downregulated the expression iNOS in OVA-challenged asthmatic mice. (a) CHR downregulated the level of iNOS mRNA in the lung tissues of OVA-challenged asthmatic mice after treatment with CHR. The mRNA level was measured by real-time PCR. (b) The expression level of iNOS in the lung tissues of OVA-challenged asthmatic mice after CHR treatment. iNOS level was detected by western blot. (c) The expression of iNOS in the airway of OVA-challenged asthmatic mice after CHR treatment detected by immunohistochemistry (IHC). Images were at 400× magnification. (d) The expression level of α-SMA in the lung tissues of asthmatic mice. The expression level of α-SMA was detected by western blot (d, left). Gray intensities of the bands were calculated and displayed in (d, right). (e) The expression of α-SMA in the airway of OVA-challenged asthmatic mice after CHR treatment detected by IHC. Images were at 400× magnification. (f) Representative photomicrographs of Masson-stained lung sections. Images were at 200× magnification. ** $p < .01$ versus Con group; ## $p < .01$ versus OVA + Vehicle group. CHR, chrysophanol; iNOS, inducible nitric oxide synthase; OVA, ovalbumin; PCR, polymerase chain reaction; α-SMA, α-smooth muscle actin [Colour figure can be viewed at wileyonlinelibrary.com]

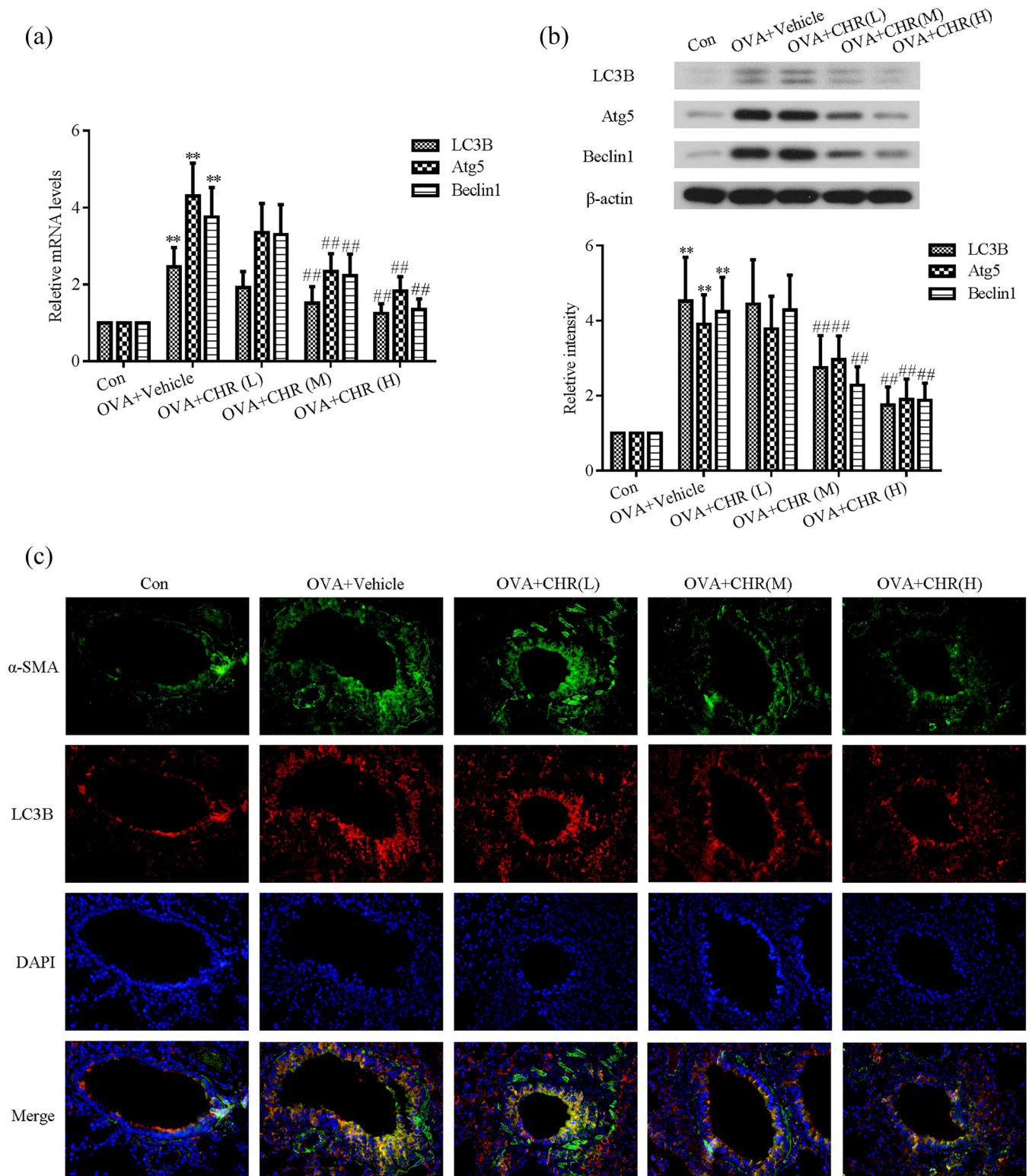


FIGURE 3 CHR inhibited autophagy in the lung tissues of OVA-challenged asthmatic mice. (a) Relative mRNA levels of autophagy-associated proteins LC3B, Atg 5, and Beclin 1 in the lung tissues of OVA-challenged asthmatic mice after CHR treatment. The mRNA levels were detected by real-time PCR. (b) The expression levels of LC3B, Atg 5, and Beclin 1 were detected by western blot. (c) The expression level of α -SMA and LC3B in the mouse airway demonstrated by immunofluorescence. Representative images of lung sections were observed at 400 $\times$ magnification. Fluorescence color: α -SMA (green), LC3B (red), and DAPI (blue). ** $p < .01$ versus Con group; ## $p < .01$ versus OVA + Vehicle group. Atg 5, autophagy-related protein 5; CHR, chrysophanol; DAPI, 4',6'-diamidino-2-phenylindole; LC3B, light chain 3 beta; OVA, ovalbumin; PCR, polymerase chain reaction; α -SMA, α -smooth muscle actin [Colour figure can be viewed at wileyonlinelibrary.com]

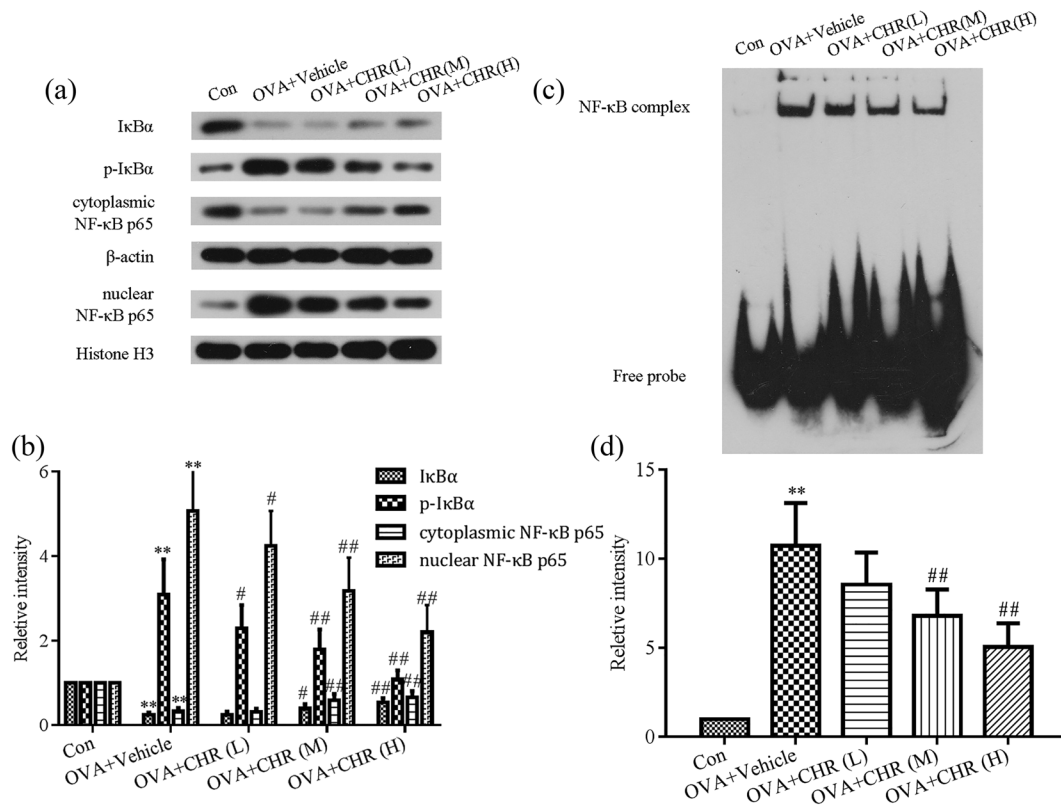


FIGURE 4 CHR inhibited NF-κB signaling pathway in OVA-challenged asthmatic mice. (a and b) The phosphorylation level of IκBα and the distribution of NF-κB in the lung tissues of OVA-challenged asthmatic mice after CHR treatment detected by western blot (a) and the gray intensities of the bands were calculated and showed in (b). (c and d) NF-κB p65-binding activity in the lung tissues of OVA-challenged asthmatic mice after CHR treatment examined by EMSA (c) and the quantitative analysis of the relative gray intensities of each group (d). ** $p < .01$ versus Con group; ## $p < .01$ versus OVA + Vehicle group. CHR, chrysophanol; EMSA, electrophoretic mobility shift assay; OVA, ovalbumin

phosphorylation and induced its degradation in the mouse lung, which was reversed by CHR to a large degree ($p < .01$). Moreover, OVA also induced the translocation of cytoplasmic NF-κB p65 sub-unit into the nucleus. CHR inhibited the nuclear translocation of p65 ($p < .01$, Figure 4a,b). We next detected whether CHR affected the DNA-binding activity of NF-κB p65 by performing electrophoretic mobility shift assay. Our results revealed that NF-κB p65 DNA-binding activity was significantly increased post-OVA treatment and was decreased by CHR ($p < .01$, Figure 4c,d). Thus, these data suggested that CHR inhibited NF-κB signaling pathway primarily through regulating the NF-κB p65 nuclear translocation as well as the NF-κB p65 DNA-binding activity. Additionally, we also observed that CHR suppressed the phosphorylation of p38 but not that of ERK (Figure S1).

3.5 | CHR suppressed the proliferation and the NF-κB signaling pathway in TNF-α-stimulated BEAS-2B cells

The proliferation of TNF-α-stimulated BEAS-2B cells was determined via MTT assay. It was found that CHR significantly suppressed TNF-α-induced proliferation of BEAS-2B cells ($p < .01$, Figure 5a). Then, we

examined total and phosphorylated IκBα protein levels, the cytoplasmic and nuclear NF-κB p65 levels in BEAS-2B cells using western blot analysis. Administration of CHR alone (either 2 or 20 μM) had no significant effect on proliferation and NF-κB signaling pathway in BEAS-2B cells (Figure S2). We noted that TNF-α augmented IκBα phosphorylation and induced its degradation ($p < .01$, Figure 5b). CHR markedly inhibited TNF-α-induced IκBα phosphorylation, suppressed IκBα degradation, and inhibited the nuclear accumulation of p65 in BEAS-2B cells ($p < .01$, Figure 5b,c). These results confirmed that CHR suppressed TNF-α-induced proliferation and inhibited the proinflammatory NF-κB pathway in BEAS-2B cells.

4 | DISCUSSION

Previous evidence has indicated that CHR exerted anti-inflammatory effects (Li et al., 2016; C. Zhang et al., 2017). Herein, we found that CHR attenuated airway inflammation and remodeling and inhibited the signaling delivery of NF-κB proinflammatory pathway in an OVA-challenged mouse model. Further, our results also demonstrated the inhibitory effect of CHR on OVA-induced autophagy in the lung tissues. Additionally, in vitro results indicated that CHR inhibited

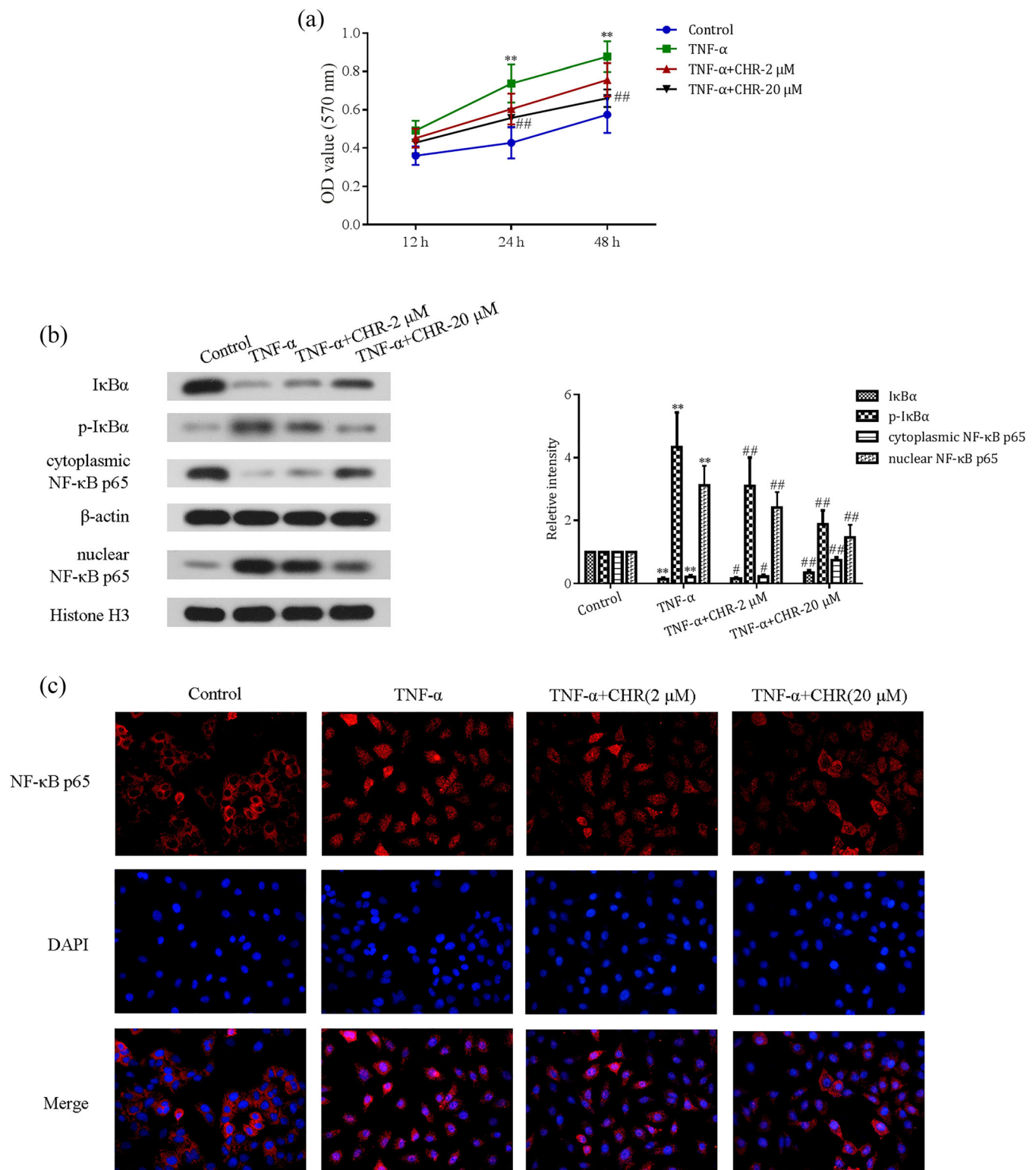


FIGURE 5 CHR suppressed cell proliferation and the NF- κ B signaling pathway in TNF- α -induced BEAS-2B cells. (a) The cell proliferation induced by TNF- α after 2- or 20- μ M CHR treatments at 12, 24, and 48 hr. BEAS-2B cells counts were detected by MTT assay. (b) CHR inhibited the activation of NF- κ B induced by TNF- α in BEAS-2B cells. The cells were first treated with CHR. TNF- α was added 24 hr later, 20 min before collecting cells. The phosphorylation level of I κ B α and the distribution of NF- κ B in cytoplasm and nucleus in TNF- α -induced BEAS-2B cells after treatment with or without CHR were detected by western blot. (c) Representative immunofluorescence images of colocalization of NF- κ B p65 (red) and DAPI (blue) in TNF- α -induced (400 $\times$ magnification). ** p < .01 versus Control cells; ## p < .01 versus TNF- α -treated cells. CHR, chrysophanol; DAPI, 4',6'-diamidino-2-phenylindole; TNF- α , tumor necrosis factor- α [Colour figure can be viewed at wileyonlinelibrary.com]

TNF- α -induced activation of NF- κ B pathway in BEAS-2B cells. CHR is a natural component in the plant, with fewer side effects; our study provides an alternative treatment strategy for patients who are not sensitive to conventional drug therapy, have adverse reactions, or have even intolerance.

It has been documented that inflammation contributes to airway remodeling, generation of ILs, and infiltration of eosinophils in asthma (Busse & Lemanske, 2001; Tagaya & Tamaoki, 2007). Airway inflammation is under the control of inflammatory mediators, neurotransmitters, and other signaling molecules, including NO (Kharitonov et al., 1994; O'Byrne et al., 2012). NO synthases convert L-arginine to L-citrulline and NO, and among these NOS, iNOS expression can be induced by inflammatory cytokines (Dweik, 2005; Nathan & Xie, 1994). iNOS predominantly expresses in inflammatory cells, such as macrophages and T cells, and it also expresses in epithelial cells (Dweik, 2005; Kobzik et al., 1993). As a matter of fact, the iNOS activity in epithelial cells is key to NO concentration in exhaled breath (Lane et al., 2004). NO produced by iNOS acts on the submucosal glands to increase mucus secretion and recruits Th1 cells to reduce the production of interferon C and Th2 cells to produce IL-4 and IL-5 (Duguet, Iijima, Eum, Hamid, & Eidelman, 2001; Xiong, Karupiah, Hogan, Foster, & Ramsay, 1999). IL-4, in turn, induces IgE production, and IL-5 recruits eosinophils into the airways (Frieri, 2005). Type 2 inflammation occurs in many patients with asthma (Fahy, 2015). An earlier study has demonstrated that the anti-inflammatory effect of CHR on asthma is associated with its inhibitory effects on IL17A generation (C. Zhang et al., 2017). Nonetheless, whether CHR affects the generation of type 2 inflammatory cytokines is unknown. We here found that CHR dose-dependently inhibited the infiltration of inflammatory cells and reduced the generation of proinflammatory IL-4, IL-5, IL13, TNF- α , and iNOS in the asthmatic lung. Although the inhibitory effects of CHR on IL-4 expression have been found in other experimental disease models (Chu et al., 2018; Lin et al., 2015), we demonstrated such antitype 2 inflammation effects of CHR in asthmatic mice for the first time.

Airway inflammation is the process of innate immunity in response to physical or physiological stress, which is associated with the activation of the NF- κ B signaling pathway (Hoesel & Schmid, 2013). Abnormal activation of NF- κ B has been observed in the airway epithelium of human and animals with asthma (Pantano et al., 2008). Activated NF- κ B signaling can trigger the generation of proinflammatory cytokines, thereby leading to the accumulation of leukocytes to the inflammatory sites (Hoesel & Schmid, 2013). We here found that CHR markedly suppressed the activation of NF- κ B p65 induced by OVA in the mouse lungs and by TNF- α in BEAS-2B cells. Our data were supported by several previous studies (S. J. Kim et al., 2010).

Chronic inflammation of the airways usually results in airway remodeling, which eventually leads to airway narrowing in asthma. In asthmatic airway, fibroblasts differentiate into myofibroblasts, which can produce extracellular matrix proteins and proinflammatory mediators (Al-Muhsen et al., 2011). By marking activated fibroblasts and myofibroblasts with α -SMA, we found that CHR reduced α -SMA-

positive cells in the lungs of asthmatic mice. Furthermore, airway remodeling is associated with aberrant epithelial cell proliferation (Alrashdan et al., 2012). Our results also showed that CHR inhibited TNF- α -induced proliferation of BEAS-2B cells in vitro. The observed anti-airway remodeling effects of CHR further suggest it as a potential therapeutic drug for asthma.

It has been reported that the autophagic markers including LC3, Atg 5, and/or Beclin 1 were upregulated in the airway of both patients with asthma and OVA-challenged mice (T. Liu et al., 2017). By double staining the mouse lung tissues with LC3B and α -SMA antibodies, we further noted that the expression of LC3B was markedly upregulated in these α -SMA-positive cells in asthmatic mice. Our findings demonstrate that autophagy is primarily augmented in activated pulmonary fibroblasts and myofibroblasts in response to OVA challenge, suggesting that autophagy induction may be associated with airway remodeling. In addition to alleviating airway inflammation and remodeling, CHR also inhibited autophagy: LC3, Atg 5, and Beclin 1 were decreased. Because inhibition OVA-induced autophagy can mitigate asthmatic inflammation and airway remodeling (T. Liu et al., 2017), it is plausible to propose that autophagy inhibition is involved in the antiasthmatic effects of CHR.

Mitogen-activated protein kinases (MAPKs), the ubiquitous signaling enzymes, are involved in asthmatic inflammation (Khorasanizadeh, Eskian, Gelfand, & Rezaei, 2017). The phosphorylation of ERK and p38 is reported to be upregulated in animal models of asthma (Dong, Davis, & Flavell, 2002; Khorasanizadeh et al., 2017). We further found that CHR effectively inhibited the phosphorylation of p38, but not that of ERK in OVA-challenged mice. Such findings suggest that CHR inconsistently affects MAPK signaling transduction in the asthmatic lung. The ongoing clinical trial of AZD-7624, a p38 MAPK inhibitor, in asthma (Khorasanizadeh et al., 2017) suggests that targeting p38 MAPK has potential in treating asthma. Thus, there is a need to elucidate the precise mechanisms underlying how CHR affects p38 signaling activation.

In conclusion, our study reveals that CHR effectively attenuates OVA-induced asthma through inhibiting airway inflammation and remodeling. These results provide experimental evidence showing that CHR has the potential to treat asthma.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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