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Cryptotanshinone attenuates allergic airway inflammation through negative regulation of NF-κB and p38 MAPK

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ABSTRACT

This study is to determine the role and mechanism of cryptotanshinone (CTS) in allergic airway inflammation. Asthma induced by OVA was established in BALB/c mice. We found increased airway hyperresponsiveness (AHR), increased inflammatory cell infiltration, elevated levels of TNF-α, interleukin-1β (IL-1β), IL-4, IL-5, IL-6 and IL-13, decreased interferon gamma (IFN-γ) in lung tissue, increased content of total immunoglobulin E (IgE), OVA specific IgE, Eotaxin, ICAM-1, VCAM-1, nuclear factor-kappaB (NF-κB) and phosphorylation of p38 MAPK in lung tissue. However, the administration of CTS significantly decreased AHR in asthmatic mice, reduced inflammation around the bronchioles and inflammatory cells around airway, regulated cytokine production, reduced the total IgE and OVA-specific IgE levels, and inhibited NF-κB activation and p38 MAPK phosphorylation. In vitro experiments in 16 HBE cells revealed that CTS attenuated CAM-1 and IL-6 expression. These results indicate that CTS alleviates allergic airway inflammation by modulating p38 MAPK phosphorylation and NF-κB activation.

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Cryptotanshinone; asthma; airway remodeling; NF-κB; p38 MAPKs

Allergic asthma is an airway chronic inflammatory disease and involved many kinds of cells and molecules, such as eosinophils, neutrophils, macrophages, cytokines, and chemokines [1]. It is featured by airway hyperresponsiveness (AHR), leukocyte infiltration, mucosal hyperplasia, and remodeling [2].

The current theory holds that the pathogenesis of asthma is related with the imbalance between Th1 and Th2 cells and their cytokines [3]. The Th2-specific cytokines, such as interleukin (IL)-4, 5 and 13, are essential inflammatory mediators for immunoglobulin E (IgE) production and humoral immune responses, and they can affect eosinophils in the airway. Th1 cytokines such as interferon (IFN)-γ and IL-12 play a key role in Th2 immune response antagonism, the cellular immune responses, and IgE production, and they could suppress the development of asthma [4]. The Th1/Th2 balance is crucial for the occurrence and development of asthma, therefore, asthma prevention and treatment may be achieved through regulating the Th1/Th2 balance [5,6].

Nuclear factor-kappa B (NF-κB) is a transcription factor widely distributed in eukaryotes and plays a key role in the transcriptional regulation of related genes such as inflammation, immune response, cell proliferation, differentiation and apoptosis, and tumor formation [7]. The human NF-κB family includes p50, p52, p65, c-Rel, and RelB. The NF-κB family typically forms homodimers or heterodimers that bind to its inhibitory complex IκB in the cytoplasm [7]. Under the stimulation of relevant factors, IκB is phosphorylated by IκB kinase (IKK), ubiquitinated subsequently and then degraded, resulting in NF-κB entry into the nucleus [8]. In the nucleus, NF-κB binds to its DNA response element to regulate the transcription of specific genes, such as the Th2 cytokines IL-4, IL-5, IL-13. Studies have shown that NF-κB is over-activated in macrophages and airway epithelial cells in asthmatic and mouse models [9], while the inhibition of NF-κB activation may be beneficial in relieving inflammatory responses [10]. For example, BAY 11–7085 is an inhibitor of NF-κB, which could inhibit NF-κB activation by inhibiting IκBα phosphorylation, and, can improve lipopolysaccharide-induced morphological changes and NO production in RAW 264.7 cells [11].

p38 MAPK was discovered in 1993 and is a 38kD protein consisting of 360 amino acids. It is a stress-activated protein kinase and is closely related to the regulation of inflammation and stress response. In recent years, more and more studies have shown that p38 MAPK may play an important role in multiple aspects of asthma [12]. The inflammation in allergic asthma was inhibited after p38 MAPK blockade, revealing the potential role of p38 MAPK as target for asthma treatment [13]. Consequently, blocking the NF-κB and MAPK may be a feasible strategy to ameliorate inflammatory responses in asthma. For example, SB239063 is an inhibitor of p38 MAPK,

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and it is reported that it can inhibit ischemia-induced IL-1β or IL-6 expression in acute lung injury [14].

Cryptotanshinone (CTS), a fat-soluble phenanthraquinone extracted from the roots of *Salvia miltiorrhiza Bunge* [15], has many biological effects, including anti-obesity, anti-inflammation [16] anti-diabetes, and anti-cancer [17–19]. It has been reported that CTS protects against IL-1β-induced inflammation in human osteoarthritis chondrocytes, ameliorates the progression of osteoarthritis in mice and attenuates rheumatoid arthritis and renal ischemia-reperfusion injury through the inhibition of NF-κB signaling pathway [20–22]. Meanwhile, CTS exerts protective effects on lipopolysaccharide-induced and radiation-induced lung injury in mice and rats [23,24]. However, there are less research of CTS on the phosphorylation of p38 MAPK and the activation of NF-κB signaling in inflammatory diseases such as asthma *in vitro*. In this study, we aim to determine whether CTS could inhibit inflammation of asthma induced by ovalbumin (OVA) and to explore the possible mechanism.

Materials and methods

Animals and experimental protocols

Thirty-five female BALB/c mice (SPF; 6–8 weeks old; 18–20 g) were from Yanbian University Health Science Center (Yanji, China). Mice were kept in standard conditions. The study complied with the code of ethics of the World Medical Association (Helsinki Declaration of 1964, as revised in 2002). The protocol was approved by the Institutional Animal Care and Use Committee of Yanbian University Medical College. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize the suffering of animals.

Establishment of OVA induced asthma model and animal treatment

The asthmatic mouse model was established as previously described [25,26]. In brief, sensitization solution (200 μL), including 1 mg adjuvant (Imject® Alum; Pierce, Rockford, IL, USA), 10 μg OVA, and saline, were intraperitoneally injected on days 1, 7 and 14. The control mice received sterile saline (200 μL) instead. From day 21 to day 23, the sensitized mice were exposed to 1% OVA (aerosol) for 30 minute. Mice in the control group received saline aerosol. On day 21 and 23, each mouse was injected with CTS (20 and 40 mg/kg body weight (BW); 98% purity; Chinese medicine monomer; Shanghai Winherb Medical Technology Co., Ltd., China) intraperitoneally at 1 h before OVA induction. Meanwhile, SB239063 (a p38 MAPK inhibitor; 0.75 mg/kg BW, Sigma), or BAY 11–7085 (a soluble inhibitor of NF-κB; 20 mg/kg BW, Sigma) was intraperitoneally administrated twice. The successful establishment of asthma model was evaluated as previous description [25–27]. At 24 h after the last OVA challenge, the mice were intraperitoneally injected with pentobarbital (50 mg/kg, i.p.; Wuhan Boster Biological Technology, Ltd., Wuhan, China). After tracheotomy and intubation, AHR measurement was performed. Then the mice were sacrificed, followed by collection of the BALF and the lung tissues for subsequent analysis.

Assessment of AHR

After tracheotomy and intubation, AHR measurement was performed. Briefly, methacholine (Mch) was administered by tail vein puncture, and airway resistance was measured by AniRes2005 animal lung function analysis system (Beijing Bestlab High-Tech Co., Ltd.) The ventilator parameters were set to a tidal volume of 6 mL/kg, a respiratory rate of 90 beats/min, and aspiration ratio at 1:1.5. Each mouse except control group was given increasing doses of methacholine at 2.5, 5, 10, 25, and 50 mg/mL, respectively.

Collection of bronchoalveolar lavage fluids (BALF) and cytospin preparations

After intubation, 1 mL of cold PBS was used to wash the lungs and the BALF was collected. Hemocytometer was used to count the number of cells. After centrifugation, the supernatants were collected for ELISA. Cell pellets were re-suspended and subjected to Diff-Quik staining (International Reagents, Kobe, Japan). The cells were then counted by two investigators independently and blindly.

ELISA

ELISA kits (R&D Systems, Minneapolis, MN, USA) were used to detect total IgE and OVA-specific IgE, IL-1β, TNF-α, IL-4, IL-13, IL-5, intracellular adhesion molecule 1 (ICAM-1), eotaxin, and vascular cell adhesion molecule 1 (VCAM-1) levels in BALF.

Histological examinations

The left lung tissues were fixed in 10% formalin, embedded in paraffin and cut into 5 μm slices. H&E staining was used to observe the inflammation of bronchus. Inflammation was graded according to previous description [28], which was 0, none; 1,< 25% of inflammatory cells; 2, 25–50%; 3, 50–75%; and 4, > 75% of inflammatory cells. PAS staining was used to assess goblet cell hyperplasia. The PAS staining-positive cells were counted. Expression of NF-kB p65 and p38 MAPK were analyzed with immunohistochemistry using

antibodies from Cell Signaling Technology (Danvers, MA, USA). Five fields of each section were observed under 200× magnification.

Western blot analysis

Cytosolic or nuclear proteins were isolated from cells or right lung tissues and protein concentrations were determined using the Bradford reagent (Bio-Rad, Hercules, CA, USA). Protein samples (30 μg) were separated using 12% SDS-PAGE, and then transferred onto a nitrocellulose membrane. The membrane was first blocked and then incubated with primary antibodies (1:1,000) overnight at 4°C. After washing, secondary antibody (1:5,000) was added and incubated for 2 h. The protein bands were analyzed with enhanced chemiluminescence detection system (Amersham Life Science, Arlington Heights, IL, USA).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA from right lung tissue of mice was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA (2 μg) was reverse transcribed to cDNA using SuperScriptII. The primer sequences used were as follows: IL-4 sense, 5′-CCA GCT AGT TGT CAT CCT GCT CTT C-3′ and antisense, 5′-GTG ATG TGG ACT TGG ACT CAT TCA TGG-3′; IL-5 sense, 5′-GAG AAG GAT GCT TCT GCA CTT GAG-3′, antisense, 5′-CCA CTC TGT ACT CAT CAC ACC AAG G-3′; GAPDH sense, 5′-AACTTTGGCAT TGTGGAAGG-3′, antisense, 5′-CATCGAAGGTGG AAGAGTGG-3′. The cDNA was amplified by PCR. PCR reaction conditions were initial denaturation at 95°C for 2 min, 40 cycles of denaturation at 95°C for 30 seconds, annealing at 52°C for 30 seconds, and extension at 72°C for 40 seconds with a final step at 72°C for 5 min. The products were separated on 2% agarose gel. The intensity of results was qualified with SynGene software and the relative mRNA level of IL-4 and IL-5 to GAPDH was calculated.

Cell culture

The human bronchial epithelial cell line (16 HBE) cells (American Type Culture Collection, Rockville, MD, USA), were cultured as previously described [29]. Cells were incubated with CTS (5 μmol/L), SB239063 (10 μmol/L) or BAY11-7085 (5 μmol/L) for 6 h. Then, the cells were stimulated with 50 ng/mL TNF-α/IL-4 for up to 24 h [30]. DMSO was used as the solvent of these reagents. Subsequently, cells were harvested for Western blot analysis.

Immunofluorescence staining

After treatment, 16HBE cells were fixed with cold paraformaldehyde for 30 min at 4°C and permeabilized with 0.5% Triton X-100 at room temperature for 15 min. After blocking for 45 min with 10% goat serum, rabbit antibody against NF-κB p65 was added and incubated overnight at 4°C. After washing, cells were probed with Alexa Fluor® 594-conjugated secondary antibody (1:1000) at room temperature in dark for 1 h. Hoechst 33342 was used to stain the nuclei solution. A Zeiss LSM 510 laser confocal fluorescence microscope (Carl Zeiss, Oberkochen, Germany) was used for image analysis.

Statistical analyses

Descriptive statistics were performed using SPSS 17.0 software. The measurement data were expressed as mean ± SD. Comparison among groups was done by One-way ANOVA analysis and LSD. $P < 0.05$ was considered statistically significant.

Results

CTS reduces mouse AHR, chemotaxis, and airway inflammation in OVA-induced asthma

We first investigated the effect of CTS on the development of AHR in mice. As shown in Figure 1(a), OVA-challenged mice had increased AHR than control mice, and this increase was partially suppressed by CTS, SB239063, or BAY 11–7085.

Compared with control group, the total inflammatory cell number and the numbers of eosinophils, neutrophils and lymphocytes in the BALF at 24 h after OVA inhalation were significantly increased (Figure 1(b)) ($P < 0.05$). After CTS, SB239063, and BAY 11–7085 treatment, the number of inflammatory cells was significantly decreased ($P < 0.05$).

In this experiment, the inflammatory cell infiltration in the airway of asthmatic mice has a certain relationship with the histological changes in the lungs of mice. OVA-challenged mice showed extensive perivascular and peribronchial inflammatory cell infiltration in the left lung. However, CTS- and BAY-treated mice showed significantly less inflammatory cell infiltration than the OVA group (Figure 1(c,d)).

In addition, the percentage of goblet cells (represented by PAS staining) that produced mucus in OVA mice was significantly higher than in control group mice. However, the application of CTS significantly reduced the number of goblet cells (Figure 1(c,e)).

The levels of Muc5AC and CLCA3 were measured by Western blot. Mice with asthma had significantly increased levels of Muc5AC and CLCA3 protein than

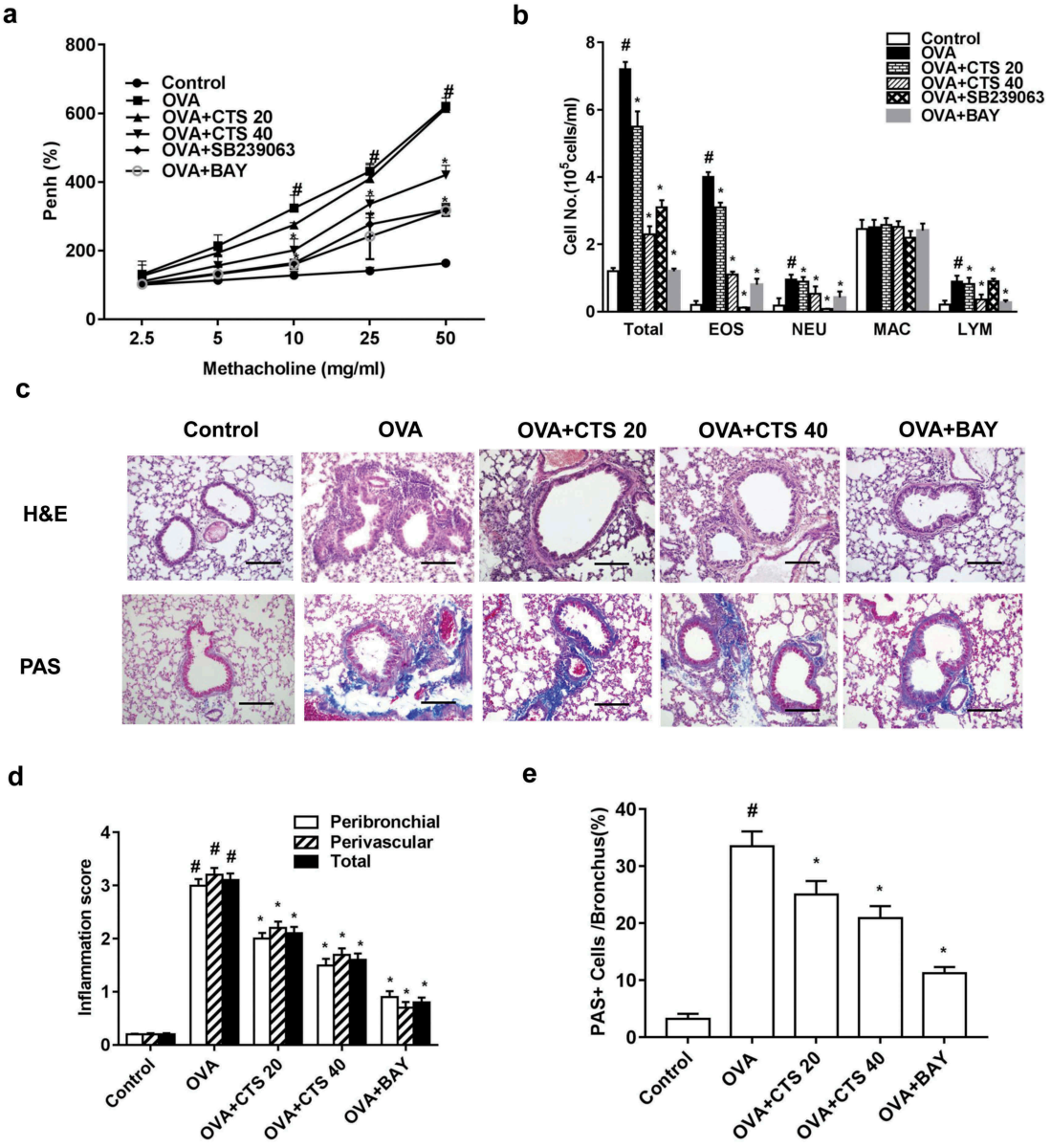

Figure 1. Analysis of lung inflammation after OVA sensitization and CTS treatment. (a) AHR was evaluated with Penh (%). (b) Cell number in BALF. EOS, eosinophil; NEU, neutrophil; MAC, macrophage; LYM, lymphocyte. (c) H&E and PAS staining results. Magnification 200 × . Scale bars = 50 μm. (d) Inflammation scores. e) Quantification of PAS positive cells. #P < 0.05 vs. Control, *P < 0.05 vs. OVA. Saline-inhaled mice administered saline (Control), OVA-inhaled mice administered saline (OVA), CTS 20 mg/kg (OVA + CTS 20), CTS 40 mg/kg (OVA + CTS 40), SB239063 (OVA + SB239063), and BAY 11–7085 (OVA + BAY).

control mice (Figure 2). Additionally, these protein levels were significantly decreased by CTS and BAY 11–7085 (Figure 2).

These results thus indicate that CTS efficiently attenuates mucus hypersecretion and allergic airway inflammation in OVA-induced asthmatic mice.

CTS decreases total IgE and OVA-specific IgE levels in the BALF of mice with OVA-induced asthma

ELISA showed that at 24 h after final OVA challenge, asthmatic mice had significantly increased levels of total IgE and OVA-specific IgE than control group mice (Figure 3(a)). However, they were significantly reduced after CTS or BAY 11–7085 treatment.

CTS reduces the levels of cytokines in lung tissues and BALF of OVA-challenged mice

ELISA results showed that the levels of TNF-α, IL-4, IL-1β, IL-13 and IL-5 in the BALF of asthmatic mice were significantly increased than control mice (Figure 3(b)). The increase in these cytokine levels was significantly reduced after CTS or BAY 11–7085 treatment. IFN-γ level was significantly decreased after OVA challenge but increased after CTS or BAY 11–7085 treatment. Consistently, WB results (Figure 3(c,d)) showed that the levels of TNF-α, IL-4, IL-1β, IL-13 and IL-5 were significantly reduced whereas that of IFN-γ was significantly increased after CTS or BAY 11–7085 treatment. In addition, we determined levels of eosinophil chemotactic factors, ICAM-1 and VCAM-1 (Figure 3(b,d)). The

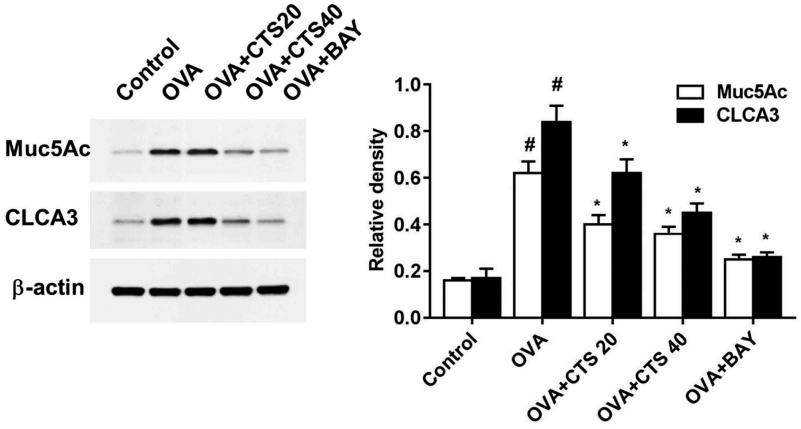

Figure 2. Analysis of Muc5AC and CLCA3 protein expression in OVA-inhaled mice. Muc5AC and CLCA3 levels in lung tissues were evaluated by Western blot at 48 h after the last OVA challenge. Representative and quantitative results were shown. #P < 0.05 vs. Control group, *P < 0.05 vs. OVA. Saline-inhaled mice administered saline (Control), OVA-inhaled mice administered saline (OVA), CTS 20 mg/kg (OVA + CTS 20), CTS 40 mg/kg (OVA + CTS 40) and BAY 11–7085 (OVA + BAY).

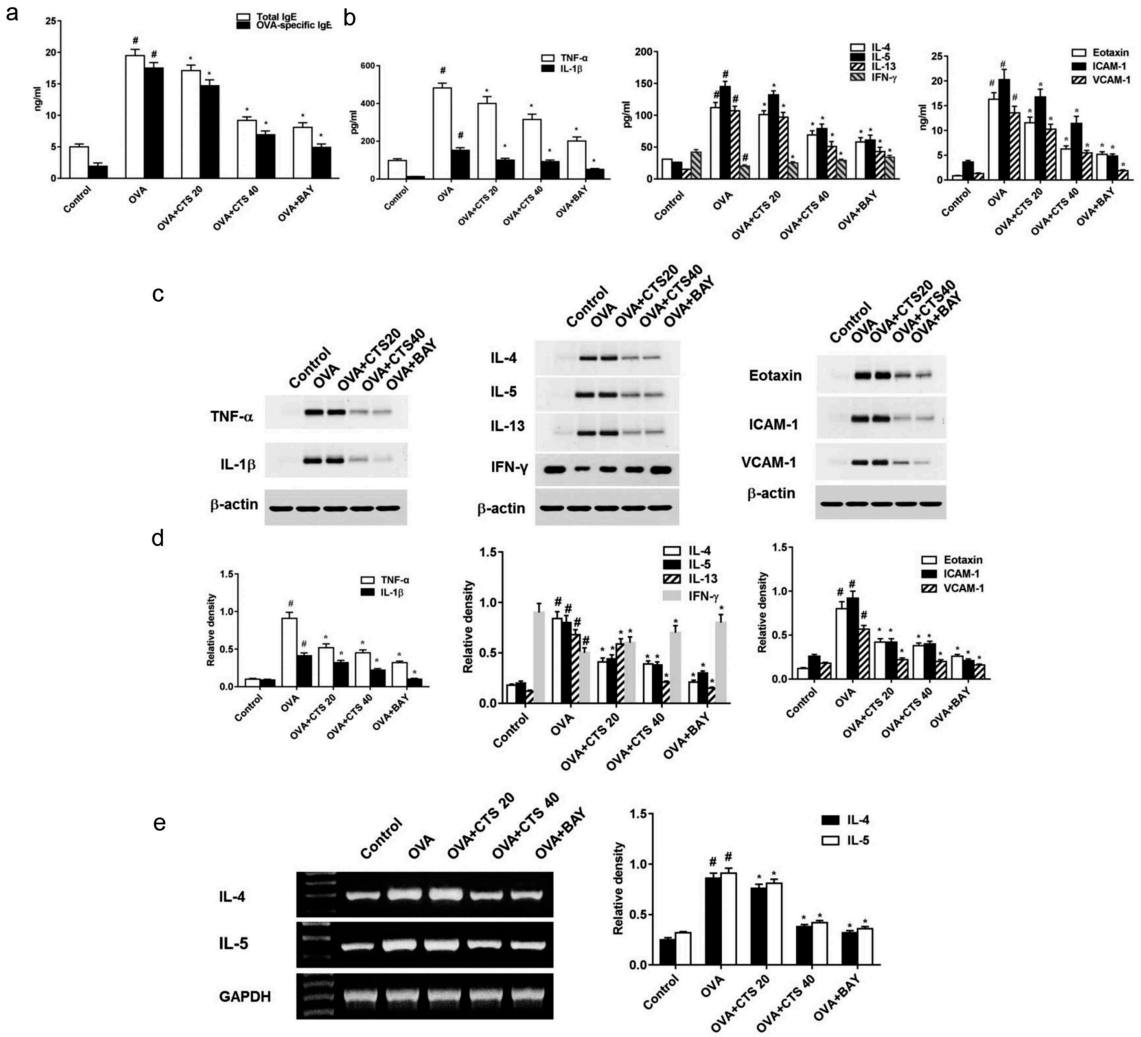

Figure 3. Analysis of IgE and cytokine levels. (a) and (b) ELISA was used to detect the levels of IgE and various cytokines in BALF. c) and d) Western blot was performed to measure cytokine expression in lung tissues. e) The mRNA expression of IL-4 and IL-5 were detected by RT-PCR. #P < 0.05 vs. Control, *P < 0.05 vs. OVA. Saline-inhaled mice administered saline (Control), OVA-inhaled mice administered saline (OVA), CTS 20 mg/kg (OVA + CTS 20), CTS 40 mg/kg (OVA + CTS 40) and BAY 11–7085 (OVA + BAY).

mRNA expression of IL-4 and IL-5 were detected by RT-PCR, and the results showed that compared with the control group, the mRNA expression levels of IL-4 and IL-5 in the lung tissue of the OVA group were significantly increased, and this effect could be inhibited by the administration of CTS or BAY 11–7085 (Figure 3(e)).

Changes in these cytokine levels were similar to changes in the above cytokines, indicating that an increase in OVA-induced cytokine levels is reversed by CTS or BAY 11–7085.

CTS inhibits OVA-induced NF-κB p65 translocation in lung tissue of mice

The nuclear translocation of the NF-κB p65 subunit in lung tissues was analyzed. Western blot showed that NF-κB p65 was decreased in the cytosols whereas increased in the nuclei of lung tissues from OVA-challenged mice. This result of NF-κB p65 nuclear translocation was verified by immunostaining of NF-κB p65 (Figure 4(b)). In contrast, after CTS or BAY treatment, NF-κB p65 nuclear translocation was suppressed. Moreover, phosphorylation and degradation of IκB-α was significantly decreased by CTS and BAY (Figure 4(c)). In conclusion, CTS may inhibit nuclear transport of NF-κB p65 subunit via stabilizing IκB-α, thus inhibiting NF-κB transcriptional activity.

CTS inhibits OVA-induced p38 MAPK phosphorylation in lung tissue of mice

The p38 MAPK was also analyzed with Western blot and immunohistochemistry. The p38 MAPK phosphorylation level was significantly increased after OVA treatment while significantly decreased after CTS or SB239063 (a p38 MAPK inhibitor) treatment (Figure 5(a)). However, p38 MAPK protein expression was not significantly changed (Figure 5(a,b)).

CTS suppresses ICAM-1 and IL-6 in 16HBE cells induced by TNF-α/IL-4

Western blot was performed to measure the expression levels of p-p38 MAPK, p38 MAPK, nuclear p65 subunit, IL-6, p-IκB-α and ICAM-1 in 16HBE cells. After TNF-α/IL-4 treatment, p65 nuclear accumulation, IκB-α phosphorylation, p38 MAPK phosphorylation, ICAM-1 and IL-6 levels in 16-HBE cells were significantly increased (Figure 6(a,b)). However, these increases were reduced by CTS significantly.

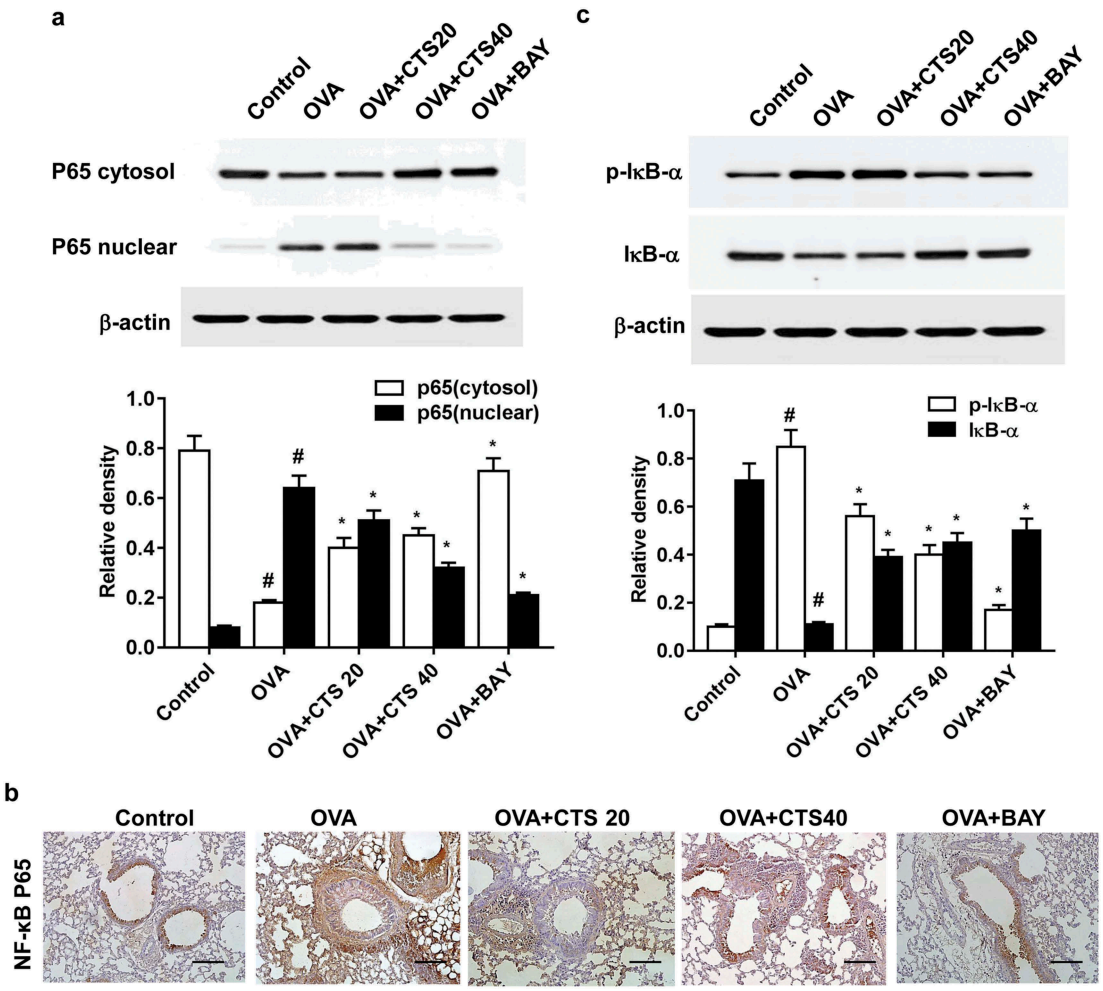

Figure 4. Analysis of NF-kB activation after OVA and CTS treatment. (a) and (c) Western blot was used to detect p65 (cytosol and nuclear), IkB-α and IkB-α phosphorylation levels. (b) Immunohistochemistry was conducted to detect NF-kB p65 expression. Bars = 50 μm. #P < 0.05 vs. Control, *P < 0.05 vs. OVA. Saline-inhaled mice administered saline (Control), OVA-inhaled mice administered saline (OVA), CTS 20 mg/kg (OVA + CTS 20), CTS 40 mg/kg (OVA + CTS 40), and SB239063 (OVA + SB239063).

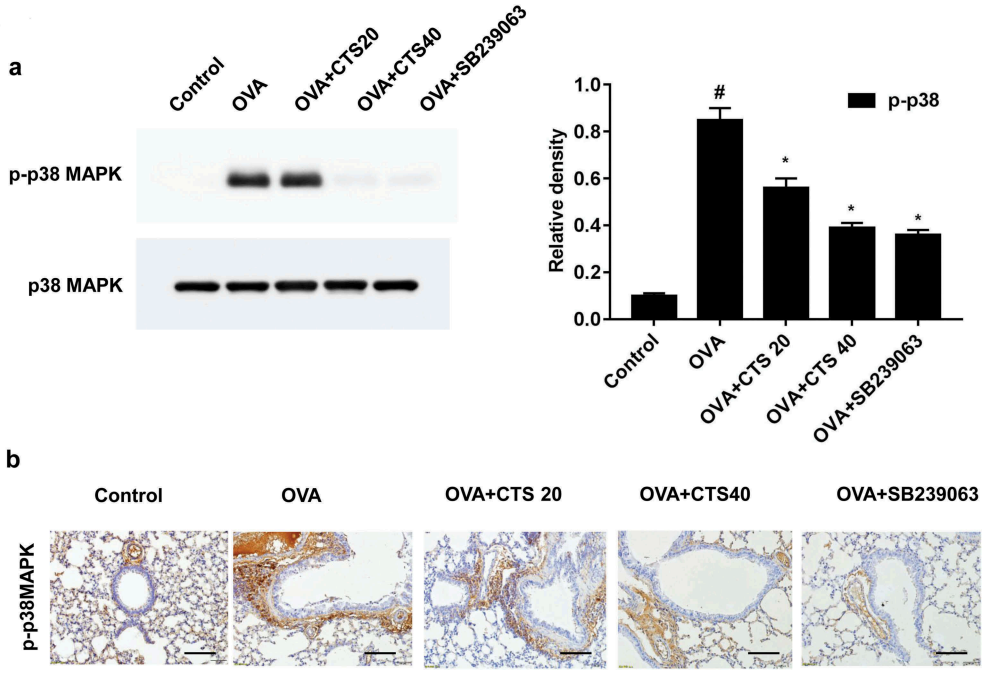

Figure 5. Analysis of p38 MAPK activation after OVA and CTS treatment. At 48 h after last OVA treatment, phosphorylated (p)-p38 and p38 levels in lung tissues were analyzed. a) Western blot results. b) Immunohistochemistry results. #P < 0.05 vs. Control, *P < 0.05 vs. OVA. Saline-inhaled mice administered saline (Control), OVA-inhaled mice administered saline (OVA), CTS 20 mg/kg (OVA + CTS 20), CTS 40 mg/kg (OVA + CTS 40), and SB239063 (OVA + SB239063).

CTS altered the NF-κB p65 location in 16HBE cells after TNF-α/IL-4 treatment

We finally investigated the effect of CTS on p65 translocation in 16-HBE cells. Immunofluorescence analysis showed that, compared with control group, the fluorescence intensity of the nuclear translocation of NF-κB p65 in 16HBE cells was markedly increased by exposure to TNF-α/IL-4 (Figure 7). However, the treatment with CTS could reduce the fluorescence intensity of the nuclear translocation of NF-κB p65.

Discussion

Airway AHR is a typical pathophysiological feature of various respiratory diseases including asthma. However, the pathogenesis is not clear. It is generally believed that airway inflammation is one of the important mechanisms leading to AHR [26]. When the airway is stimulated by allergens and other factors, inflammatory cells and mediators are activated and released, resulting in impaired airway epithelium and intraepithelial nerves and airway AHR. Although a variety of inflammatory cells are involved in the development of AHR, the effects of eosnophil activation and infiltration are ultimately produced as the final pathway [28], so eosnophils are crucial in the formation of AHR.

It is reported that the inflammatory response plays a crucial role in the occurrence of asthma. In the airway of patients with acute asthma, inflammatory cells are significantly increased and extensively infiltrated, such as eosnophils, neutrophils, and lymphocytes, et al. These cells produce and release a variety of cytokines, such as Th1 cytokine IFN-γ, Th2 cytokines IL-4, IL-5 and IL-13, which directly affect the occurrence of asthma [1]. Besides, some adhesion molecules, including ICAM-1 and VCAM-1, are involved in the inflammatory reaction. They are immunoglobulins produced on the cell surface, which mediate the adhesion between cells and cells and between cells and vascular endothelium in the inflammatory response and are the basis of inflammatory reactions. ICAM-1 and VCAM-1 also promote transmembrane metastasis of eosinophils during airway inflammation [31]. In the meanwhile, they could increase the number of goblet cells around the airway epithelium, increase the mucus secretion, and collagen deposition under the airway. Mucin is the main protein component in mucus, and its coding gene is gene MUC. So far, 11 kinds of mucin genes have been discovered. Previous studies have shown that MUC5AC is most expressed in the airway and plays an important role in airway mucus hypersecretion [29,30].

Chemokine cytokines are cytokines with chemotactic function. Eotaxin is highly specific for eosinophils and is a potent eosinophil chemotactic agent. It is reported that Eotaxin and its receptor CCR3 are involved in asthma and they play an important role in the pathogenesis of asthma [32]. In recent years, the central role of airway epithelium in asthma pathogenesis has attracted much attention [33]. In asthmatic patients and mouse models of allergic airway disease, hCLCA1 and its mouse-like homolog, mCLCA3, are

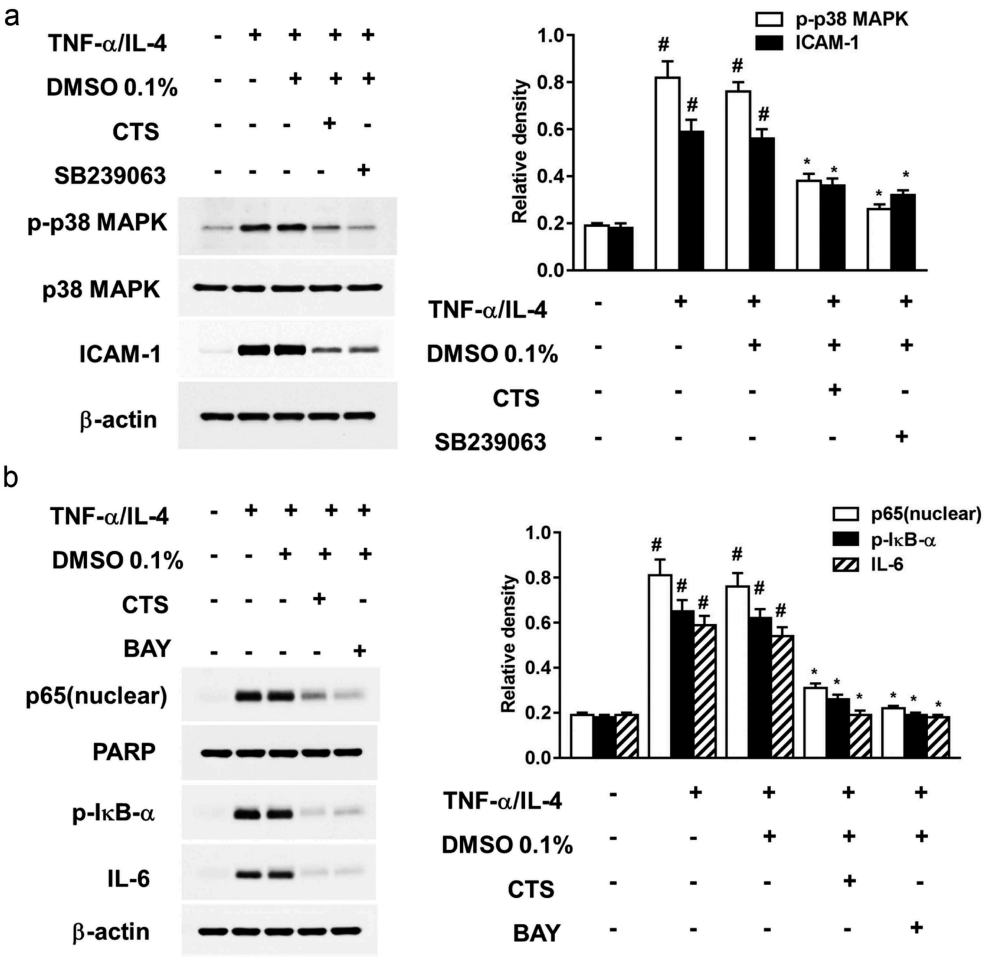

Figure 6. Analysis of p38 MAPK and other signaling intermediates levels in 16HBE cells after TNF-α/IL-4 treatment. (a) and (b) Western blotting was used to detect the levels of phosphorylated (p-) p38 MAPK, ICAM-1, nuclear NF-kB p65, phosphorylated (p-) IkB-α, and IL-6 in TNF-α/IL-4–treated 16HBE cells. 16HBE cells were pretreated with CTS (CTS, 5 μmol/L), SB239063, or BAY 11–7085 for 6 h prior to TNF-a/IL-4 treatment. #P < 0.05 vs. the untreated group, *P < 0.05 vs. the group treated by TNF-α/IL-4.

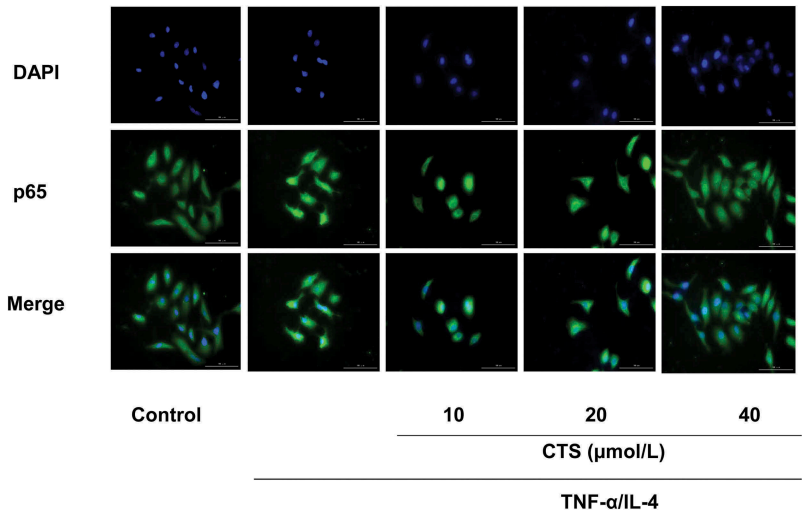

Figure 7. The effects of CTS on the location of NF-kB p65 were detected by immunofluorescence staining under a laser confocal fluorescence microscope. Magnification, 200X, scale bar: 100 μm.

one of the genes that are significantly induced to express in the airway epithelium [34]. Nakanishi have shown that by using antisense RNA technology to down-regulate the expression of mCLCA3 in allergic mice, mucus secretion of mice airway, airway AHR and eosinophil airway inflammation can be inhibited [35]. Another study also showed that mCLCA3 can increase the number of goblet cells and may regulate lung tissue inflammation by altering the migration pattern of inflammatory cells [36].

TNF-α is also an important pro-inflammatory cytokine in airway inflammation, and its interaction with endothelin plays an important role in the formation of AHR [37]. When patients inhale allergens, TNF-α induces mast cells to release inflammatory mediators, causing AHR and airway stenosis.

IL-1β is involved in the development of inflammatory reactions. IL-1β can promote the adhesion of eosinophils to vascular endothelial cells, facilitate the recruitment and infiltration of eosinophils into the bronchial mucosa, stimulate the degranulation of monocytes and eosinophils, and enhance their cytotoxic effects, which induce asthma [38].

IL-6 is secreted by the novel Th cell subset Th17, which is both an important pro-inflammatory factor and involved in the differentiation of $CD4^+$ T cells, and is a trigger for the induction of airway inflammation [39].

In this study, in asthmatic mice, CTS significantly inhibited airway AHR; reduced the number of total inflammatory cells, eosinophils, lymphocytes, and neutrophils in BALF; increased the infiltration of inflammatory cells around the trachea and perivascular cells; inhibited airway mucus secretion and the number of goblet cells; in the meanwhile, CTS upregulated the expression of Th1 cytokine IFN-γ in BALF, reduced the expression of Th2 cytokines IL-4, IL-5 and IL-13 in BALF, and decreased the levels of serum total IgE, OVA-specific IgE, TNF-α, IL-6 and IL-1β. The changes of IFN-γ and IL-4 after inhibition of NF-κB signaling pathway are consistent with previous studies [40–42]. On the contrary, Aronica et al. [43] reported that inhibition of p65 suppressed IFN-γ rather than IL-4 in vivo. In their study, the level of IL-4 and IFN-γ were detected from the cell suspension extracted from the draining lymph nodes of individual IkBa(DN) transgenic mice and wild-type littermates, which is different from our experimental conditions. Therefore, we believe that the inconsistency may be due to different experimental conditions. Further studies are needed to clarify this inconsistency. In a word, these findings suggest CTS has a certain therapeutic effect on allergic airway inflammatory diseases.

The inflammatory response of asthma has a certain relationship with the cellular signaling pathway, but the specific mechanism is still not clear. NF-κB is closely related to various inflammatory responses including asthma [44]. In normal conditions, NF-κB binds to its inhibitory protein IκBα in the cytoplasm. When cells are stimulated by related factors, IκBα can be phosphorylated and then degraded. With nuclear localization sequence exposed, NF-κB enters into the cell nucleus to bind to specific sites of the target gene, promoting transcription of related genes [45].

MAPKs are important signal transduction pathways in cells, and they are widely distributed in the cytoplasm. They can transmit extracellular stimuli into the nucleus and regulate cell proliferation, differentiation and stress. A common feature of this family to transmit information is the highly conserved three-level kinase cascade signaling: extracellular stimulation activates MKKK (MAP kinase kinase) through the corresponding pathways, which in turn activates MKK (MAP kinase kinase) and then activate MAPK through the simultaneous phosphorylation of two sites, threonine (T) and tyrosine (Y). Activated MAPK can phosphorylate various transcription factors, cytoskeleton-associated proteins, enzymes and other substrates to regulate a variety of cellular physiological functions [46]. MAPK subfamilies such as extracellular regulated kinase (ERK), c-Jun N-terminal kinase (JNK), p38 and ERK5 have been cloned and identified in mammals. Among them, p38 MAPK can regulate cellular immune regulation and inflammatory response under stress conditions. A number of studies have demonstrated that p38 MAPK is involved in the localization and migration of mast cells and eosinophils, the development, differentiation, maturation and activation of lymphocytes [47–49], suggesting an important role of p38 MAPK in asthma pathogenesis.

Phosphorylated p38 MAPK (p-p38 MAPK) phosphorylates mitogen and stress-activated protein kinase 1 (MSK1). Phosphorylated MSK1 (p-MSK1) phosphorylates NF-κB subunit RelA (p65) at Ser276 and activates NF-κB [50]. Activated NF-κB could regulate the expression of various inflammatory molecules, such as IFN-γ, IL-4, IL-5, IL-13, and play the role of inflammatory regulation by regulating Th1/Th2 cytokine levels. In our study, the inhibitor of p38 MAPK SB239063 and the inhibitor of NF-κB BAY 11–7085 were used as positive controls. The results of this experiment showed that CTS effectively inhibited the phosphorylation of p38 MAPK in inflammatory cells in airway epithelial cells and lung tissues, and inhibited the expression of NF-κB p65 in the nucleus of lung tissue. Subsequently, in order to further verify whether CTS directly regulates NF-kB activity in allergic reactions, we performed *in vitro* experiments using 16 HBE cells. We found that p38 MAPK phosphorylation and NF-κB p65 nuclear translocation in 16 HBE cells were promoted by TNF-α/IL-4. However, the use of inhibitors (SB239063 and BAY 11–7085) attenuates these effects, and the same experimental results also appeared in immunofluorescence staining.

Therefore, the results of this experiment indicate that CTS has obvious anti-asthmatic effects, and the mechanism may be associated with the down-regulation of p38 MAPK/NF-κB signaling pathway to increase Th1 cytokine, and decrease Th2 cytokine secretion.

Author contributions

All authors participated in the design, interpretation of the studies and analysis of the data and review of the

manuscript. Junfeng Li and Mingyu Zheng conducted the experiments, analyzed the data, and wrote the manuscript. Chongyang Wang, Jingzhi Jiang, and Chang Xu provided help in animal model establishment. Li Li and Liangchang Li supplied critical reagents. Yongde Jin conceived the idea, designed the study and helped to revise the manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Data availability

The data that support the findings of this study are available on request from the corresponding author.

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