

Bergapten ameliorates combined allergic rhinitis and asthma syndrome after PM2.5 exposure by balancing Treg/Th17 expression and suppressing STAT3 and MAPK activation in a mouse model

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ABSTRACT

Combined allergic rhinitis and asthma syndrome (CARAS) causes chronic respiratory inflammation in allergic individuals. Long-term exposure to particulate matter 2.5 (PM2.5; particles 2.5 μm or less in diameter) can aggravate respiratory damage. Bergapten (5-methoxysporalen) is a furocoumarin mostly found in bergamot essential oil and has significant antioxidant, anticancer, and anti-inflammatory activity. This study created a model in which CARAS was exacerbated by PM2.5 exposure, in BALB/c mice and explored the potential of bergapten as a therapeutic agent. The bergapten medication increased ovalbumin (OVA)-specific immunoglobulin (Ig) G2a level in serum and decreased OVA-specific IgE and IgG1 expression. Clinical nasal symptoms diminished significantly, with weakened inflammatory reaction in both the nasal mucosa and lungs. Furthermore, bergapten controlled the T helper (Th)1 to Th2 ratio by increasing cytokines associated with Th1-like interleukin (IL)-12 and interferon gamma and decreasing the Th2 cytokines IL-4, IL-5, and IL-13. Factors closely related to the balance between regulatory T cells and Th17 (such as IL-10, IL-17, Forkhead box protein P3, and retinoic-related orphan receptor gamma) were also regulated. Notably, pro-inflammatory cytokines IL-6, IL-1 β , and tumor necrosis factor-alpha were reduced by bergapten, which suppressed the activation of both the signal transducer and activator of transcription 3 signaling pathway and the mitogen-activated protein kinase signaling pathway. Therefore, bergapten might have potential as a therapeutic agent for CARAS.

1. Introduction

Allergic rhinitis (AR) is a non-infectious inflammatory illness of the nasal mucosa with recognizable clinical symptoms such as watery nose, nasal congestion, nasal itching, and sneezing [1]. Asthma is another chronic respiratory illness and involves airway reactions to different triggers that then cause inflammation and narrowing of the airways to produce symptoms such as difficulty breathing, wheezing, chest tightness, and coughing [2]. AR and asthma are common airway diseases with a high prevalence in populations worldwide [3]. Asthma develops in roughly 40 % of people with AR, and 70–80 % of asthmatic patients also have AR [4]. The condition known medically as combined allergic

rhinitis and asthma syndrome (CARAS) occurs when an individual experiences allergy symptoms in both the upper and lower respiratory tracts. Nasal and bronchial mucosal allergic inflammation are immunological and pathological alterations caused by CARAS [5,6].

The type 2 T helper (Th2) immune response mechanism, which triggers the inflammatory response, leads to overexpression of several cytokines, including interleukin (IL)– 4, IL-5 and IL-13, as well as to release of immunoglobulin (Ig) E, which causes infiltration and proliferation of inflammatory cells in the mucosal epithelium in the airway [7]. According to earlier research, an imbalance between Th1 and Th2 is essential for the onset of inflammation. Furthermore, several recent studies have shown that the development of allergic airway

Abbreviations: AR, allergic rhinitis; BG, bergapten; BALF, bronchoalveolar lavage fluid; CARAS, combined allergic rhinitis and asthma syndrome; Dex, dexamethasone; ERK, extracellular signal-regulated kinase; FOXP3, Forkhead box protein P3; H&E, hematoxylin and eosin; IFN- γ , interferon gamma; IL, interleukin; Ig, immunoglobulin; JNK, c-Jun amino-terminal kinase; MT, Masson trichrome; MAPK, mitogen-activated protein kinase; NALF, nasal lavage fluid; OVA, ovalbumin; PM2.5, particulate matter smaller than 2.5 μm ; PAS, periodic acid–Schiff; p-STAT3, phosphorylated (Tyr705) STAT3; ROR γ , retinoic-related orphan receptor gamma; STAT3, signal transducer and activator of transcription 3; TNF- α , tumor necrosis factor-alpha; Th, T helper; TB, toluidine blue.

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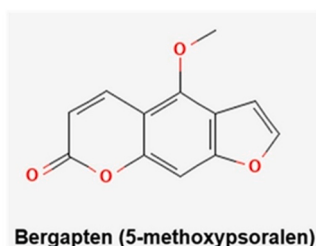


Fig. 1. Molecular formula of bergapten (5-methoxypsoralen).

inflammatory diseases can depend heavily on Th17 cells and regulatory T cells (Treg) [8,9]. Specifically, Tregs significantly affect both auto-immune tolerance and inflammatory responses [10]. IL-17 is secreted by Th17 cells, is closely related to eosinophilic airway inflammation, and mediates airway neutrophilic inflammation [11,12]. Additionally, the mitogen-activated protein kinase (MAPK) pathway is an essential signaling mechanism for a number of physiological processes, including modulation of inflammatory responses [13]. The role of the MAPK pathway in the development and maintenance of airway inflammation in asthma has been widely reported [14,15]. It has been established that MAPKs have a role in the pathogenesis of asthma-like Th2 inflammation; their inhibition of the extracellular signal-regulated kinase (ERK)

pathway can reduce OVA-induced allergic airway inflammation by suppressing the activation of IL-5R and eotaxin receptor in eosinophils [16]. c-Jun amino-terminal kinase (JNK) and p38 pathways typically become active in response to cytokines or stress [15,17,18]. Inflammation-related transcription factors are activated by the phosphorylated MAPK pathway (ERK, JNK and p38), which also controls the expression of associated genes, increasing the production of various cytokines and chemokines to participate in the inflammatory reaction [19].

The severity of air pollution is increasing as industrialization spreads worldwide. Human health is seriously threatened by particulate matter (PM) suspended in the air. PM_{2.5} (particulate matter 2.5) denotes airborne particles smaller than 2.5 μm in diameter. As particulates that small can infiltrate deeply into the lungs, PM_{2.5} is easily inhaled by humans and can cause oxidative stress and inflammation and lead to a variety of lung disorders [20,21]. In recent years, PM_{2.5} has emerged as a significant global air pollutant that accounts for an ever-increasing share of inhalable PM year after year. Unsurprisingly, PM_{2.5} has emerged as a key aggravating factor in AR, asthma, and other lung disorders [22,23].

A bergapten called furocoumarin (5-methoxypsoralen, PubChem CID: 2355, Fig. 1) is primarily extracted from bergamot essential oil. Existing research has demonstrated that bergapten possesses significant

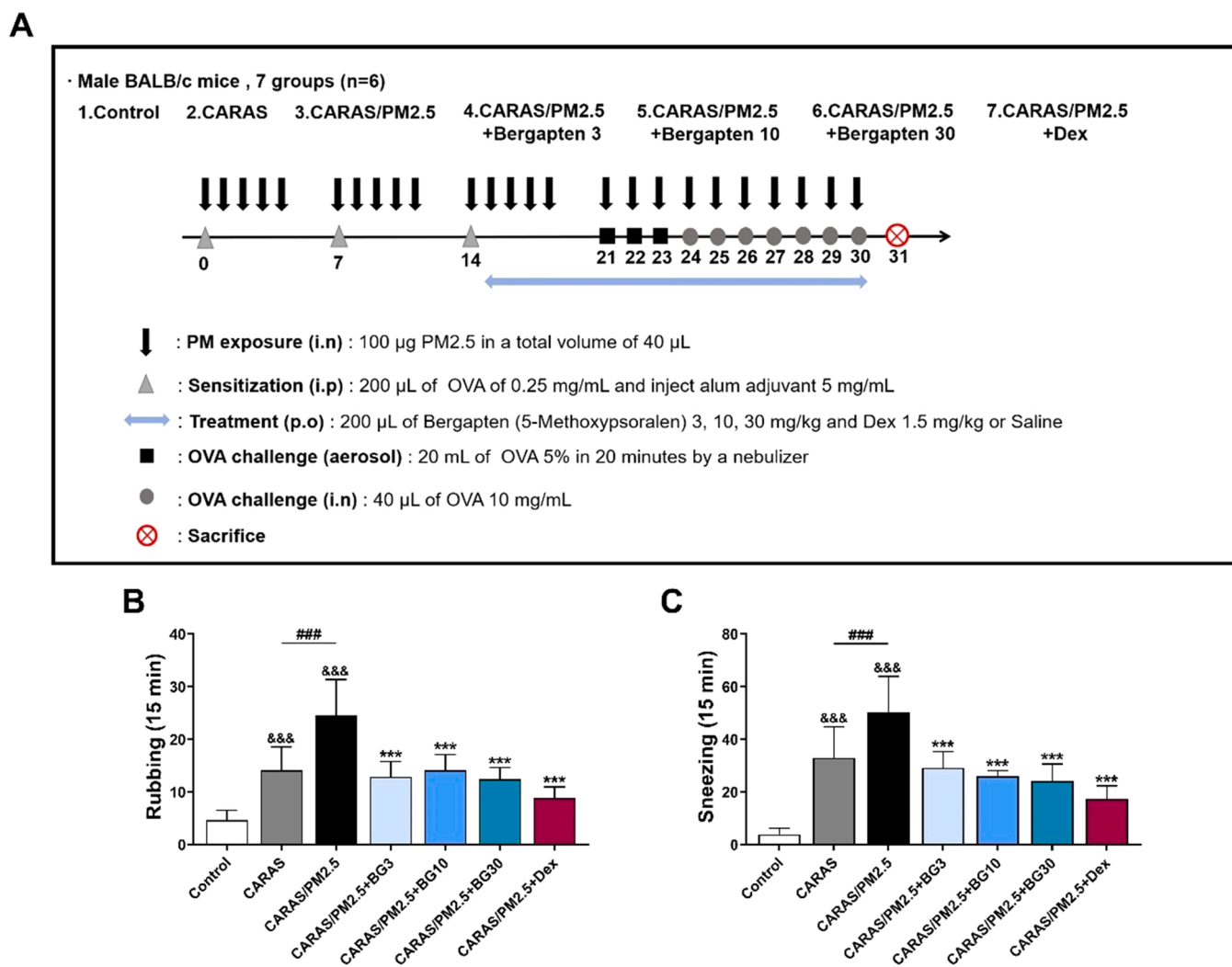


Fig. 2. Bergapten improved the nasal symptoms of CARAS/PM_{2.5} mice. (A) Establishment of the CARAS/PM_{2.5} mouse model. (B) Nasal rubbing. (C) Nasal sneezing. (All groups, n = 6) Each data point represents the mean $\pm$ SD. Significant deviations from the control group, &&& $P < 0.001$; from the CARAS group, ### $p < 0.001$; from the CARAS/PM_{2.5} group, *** $P < 0.001$.

anti-inflammatory, antioxidant, and anticancer properties [24–27]. Bergapten inhibited tail-cutting-induced inflammation in zebrafish models [28], and also had significant effects in murine models of acetic acid-induced colitis [29]. Bergapten has been found to lessen the hypersensitivity caused by lipopolysaccharide by decreasing mast cell degranulation in associated pathways [30]. Additionally, bergapten was used in ethanol-induced ulcer models as a gastroprotective agent [31]. Also, bergapten inhibited the production of the pro-inflammatory cytokines IL-6, IL-1 β , and tumor necrosis factor- α (TNF- α) [24,32–34]. Bergapten inhibited the production of TNF- α and IL-6 by PBMCs stimulated with lipopolysaccharide [24], and significantly ameliorated interleukin-1 β -induced expression of inflammatory cytokines and mediators in Osteoarthritis model [32].

In this study, we investigated the anti-inflammatory properties of bergapten by monitoring alterations in the lower and upper respiratory tracts of CARAS/PM2.5-sensitized mice. We calculated the incidence of scratching and sneezing, calculated the total cell count and number of distinct inflammatory cells in nasal lavage fluid (NALF) and bronchoalveolar lavage fluid (BALF), compared histopathologic alterations in nasal mucosa and lung tissues, and assessed transcription factors and cytokines related to Th1, Th2, Treg, and Th17 cells. Finally, we assessed how bergapten affected the signal transducer and activator of transcription 3 (STAT3) and MAPK pathways.

2. Materials and methods

2.1. Materials

PM2.5 (Urban particulate matter NIST1648A, Sigma-Aldrich, St. Louis, MO, USA) was ordered from Sigma-Aldrich and prepared by dissolution in sterile saline solution. Bergapten (5-methoxypsoralen), dexamethasone (Dex), and ovalbumin (OVA; Grade VI) were procured from Sigma-Aldrich (St. Louis, MO, USA). Aluminum hydroxide was acquired from Thermo Fisher Scientific (Rockford, MD, USA).

2.2. Animals

Damool Science (Dae-jeon, Korea) provided male BALB/c mice that were five weeks old. Prior to the experiment, the mice were kept in typical laboratory settings for one week at $23 \pm 3^\circ\text{C}$, $55 \pm 10\%$ relative humidity, and 12 h light/dark cycles. Requirements set by the Institutional Animal Care and Use Committee of Jeonbuk National University Medical School (JBNU 2021–0115) were followed in all aspects of the procedures using animals in experiments.

2.3. PM2.5-exacerbated mouse model of CARAS: establishment and treatment

Seven groups of mice ($n = 6$) were created: control group, CARAS group, CARAS/PM2.5 group, bergapten (3, 10, and 30 mg/kg) treatment groups, and Dex 1.5 mg/kg treatment group. In all groups, 100 μg of PM2.5 in a 40 μL total volume was administered intranasally from days 0–30. On days 0, 7, and 14, sensitization of the mice was performed intraperitoneally using 0.25 mg/mL OVA and 5 mg/mL aluminum hydroxide. The mice in those groups were treated orally with 200 μL of 3, 10, or 30 mg/kg of bergapten or 1.5 mg/kg of Dex from days 15–30 (1 h before OVA challenge) by using the feeding needle (11Street Co., Ltd, Jung-gu, Seoul, Korea). On days 21 through 23, the mice were exposed to an aerosol containing 5 % OVA, and from days 24 through 30, the mice were treated daily with 10 mg/mL of OVA intranasally. The same amount of saline solution was administered to the normal control group that did not receive sensitization therapy. The mice were sacrificed one day following the final intranasal OVA challenge (Fig. 2A).

2.4. Allergic symptoms

Nasal rubbing and sneezing without anesthetic were observed during a 15-minute period on day 30, following the final 10 mg/mL OVA intranasal challenge, and the frequencies were calculated by blinded observers.

2.5. Collection of blood samples

Diethyl ether anesthesia was administered to all mouse groups the day following the last OVA intranasal challenge. After collecting blood samples from the retro-orbital plexus, the mice were euthanized using diethyl ether. To obtain serum, samples of blood were centrifuged at 10,000 rpm for 10 min at 4°C . Then the serum was preserved at -80°C for further use.

2.6. Collection and examination of NALF and BALF

A tracheotomy was used to collect NALF 24 h after the last intranasal challenge, and an 18-gauge catheter was used to administer 1 mL of normal saline into the nasal canal. BALF was sampled by cannulating the lower trachea and irrigating the lungs. NALF and BALF were centrifuged (Gyro 1730 MR, Gyrozen, Gyeonggi-do, Korea) at 10,000 rpm for 10 min at 4°C . For further examination, the supernatants were gathered and stored at -80°C . A hemocytometer was used to acquire both total and differential inflammatory cell counts. Next, 150 μL each of NALF and BALF was centrifuged for 10 min at 1000 rpm and 4°C using a Cytospin device (Centrifuge 5403 R, Eppendorf, Germany), and the cells were mounted on a slide. Slides stained with Diff-Quik reagent (Wakihama-Kaicafeinendori, Chou-Ku, Kobe, Hyogo, Japan) enabled counting of differential inflammatory cells under an optical microscope. Every procedure followed manufacturer instructions.

2.7. Determination of the serum concentrations of OVA-specific IgE, IgG1, and IgG2a

The expression of OVA-specific IgE (BioLegend Inc, San Diego, CA, USA), anti-OVA IgG1 (Cayman Chemical, Ann Arbor, MI, USA), and mouse anti-OVA IgG2a (Chondrex Inc, Woodinville, WA 98072, USA) in serum was evaluated using ELISA kits according to manufacturer instructions.

2.8. Histopathological examination of nasal tissue

For nasal histopathological examination, the acquired tissue samples were preserved for three days in 10 % neutral buffered formalin and decalcified in Calci-Clear Rapid histological decalcifying agent (National Diagnostics, Atlanta, GA) on a shaker at room temperature for two days. The tissues were embedded in paraffin, cut into 4- μm slices, and stained with hematoxylin and eosin (H&E; Sigma-Aldrich, St. Louis, MO, USA) to observe thickening of nasal mucosal tissue. Goblet cell proliferation was examined using the periodic acid-Schiff (PAS; Sigma-Aldrich; St. Louis, MO, USA) technique. Mast cell migration into the mucosae of the epithelium was evaluated using toluidine blue (TB; Sigma-Aldrich; St. Louis, MO, USA). Giemsa stain (Sigma-Aldrich; St. Louis, MO, USA) was applied to study eosinophil aggregation. Nasal mucosa thickness was measured on an NIS-Elements Viewer (Nikon Instruments Inc, Melville, NY, USA). With one slide of three mice in each group, Fiji software computed the mast cell, eosinophil, and goblet cell numbers in the nasal mucosae.

2.9. Histopathological examination of lung tissue

Samples of lung tissue were shaken for three days at room temperature in 10 % formalin solution for fixation and then were embedded in paraffin. Next, 4- μm slices were stained with H&E to assess

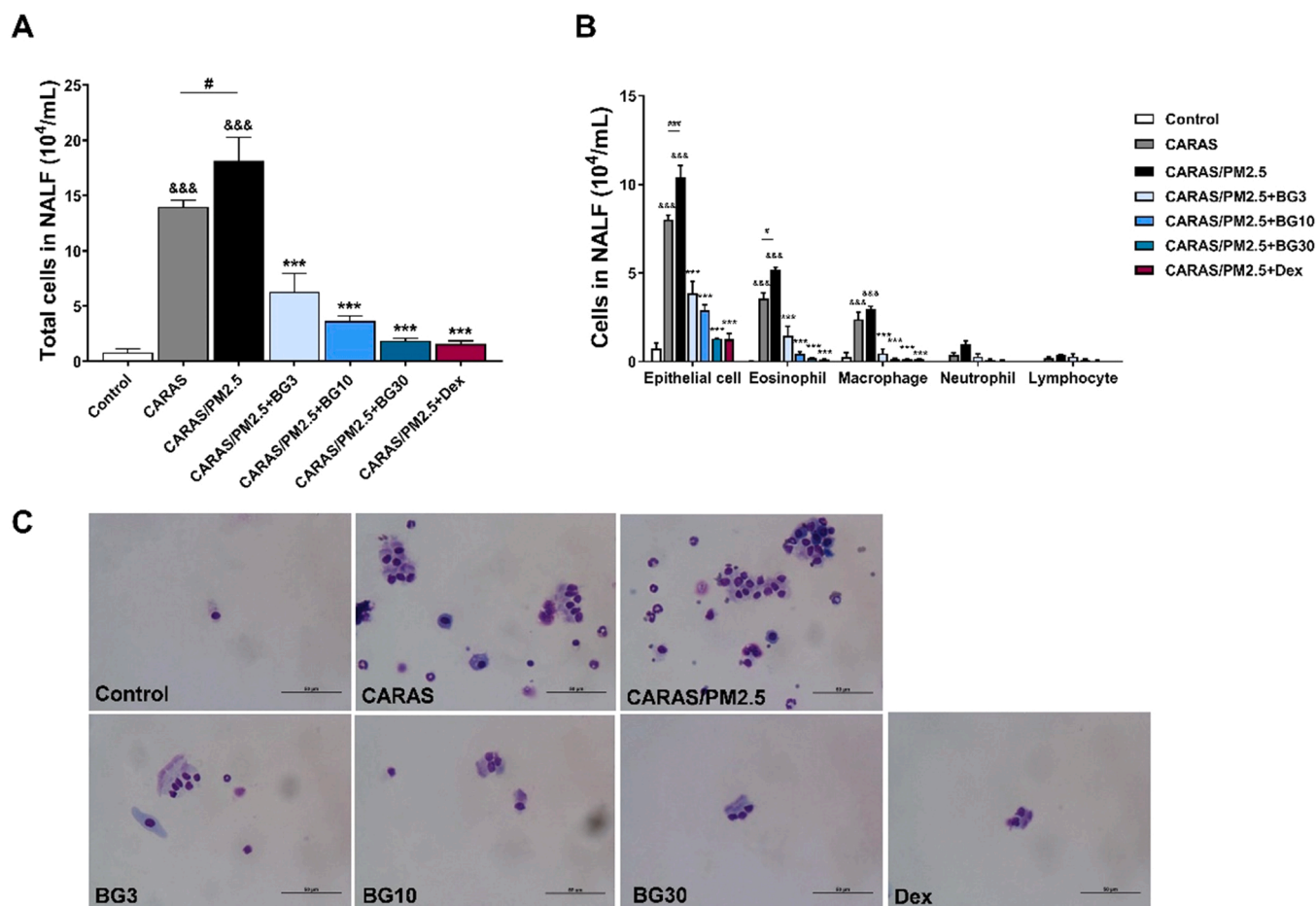


Fig. 3. Infiltration of total cells and differentiated inflammatory cells in NALF was decreased by bergapten. (A) Total cells. (B) Differentiated inflammatory cells. (C) Diff-Quik staining. Estimations of the quantities of both total and differentiated inflammatory cells by a hemocytometer. All data (n = 6 per group) are shown as mean $\pm$ SD. Significant deviations from the control group, &&& $p < 0.001$, & $p < 0.05$; from the CARAS group, ### $p < 0.001$; from the CARAS/PM2.5 group, *** $p < 0.001$. Scale bar = 50 μ m.

inflammatory responses. Lung inflammation was graded based on degree of inflammatory cell infiltration into the bronchus [35]. PAS staining was used to illustrate hyperplasia of goblet cells, TB staining showed mast cell movement, and Masson trichrome stain (MT; Sigma-Aldrich, St. Louis, MO, USA) visualized lung tissue collagen fiber deposition. The PAS-positive area, mast cell count, and area positive for collagen deposition were calculated by the Fiji program using slides for three mice in each group.

2.10. Measurement of Th1, Th2, Th17, and Treg cytokines; Th17- & Treg-associated transcription factors ROR γ and FOXP3; and inflammatory cytokines

In this experiment, various cytokines in the supernatant of NALF and BALF were evaluated using corresponding ELISA kits. R&D System (Minneapolis, MN, USA) provided ELISA kits for the pro-inflammatory cytokines (TNF- α , IL-6, and IL-1 β), cytokines related to Th1 (IFN- γ and IL-12) and Th2 responses (IL-4, IL-5, and IL-13), and cytokines linked with Th17 (IL-17) and Treg responses (IL-10). In addition, the Th17-related transcription factor retinoic-related orphan receptor gamma (ROR γ ; Finetest, Wuhan, CHINA) and Treg-related transcription factor Forkhead box protein P3 (FOXP3; Mybiosource, San Diego, CA, USA) in NALF and BALF from each mouse were assayed using ELISA kits. Each step was carried out as the manufacturer suggested.

2.11. Immunohistochemistry (IHC)

The paraffinized sections of nasal tissue (4- μ m) were dewaxed. IHC was conducted following the instructions in the IHC kit (Abcam, Cambridge, UK). Sample sections were incubated overnight with the primary antibody phosphorylated (Tyr705) STAT3 (p-STAT3, Cell Signaling Technology, Danvers, MA, USA) under proportionate dilution at 4 $^{\circ}$ C. Following subsequent incubation with anti-rabbit IgG-HRP (Abcam, Cambridge, UK), color was developed with diaminobenzidine chromogen solution. A representative image from each slide was viewed through an optical microscope.

2.12. Western blotting

Western blotting was used to assess the expression of Th17-transcription factors, including ROR γ t (Thermo Fisher Scientific, Waltham, MA, USA), p-STAT3, and STAT3 (Cell Signaling Technology, Danvers, MA, USA), the Treg-transcription factor FOXP3 (Abcam, Cambridge, UK), and MAPK signaling pathway-associated proteins such as p38, p-p38, ERK, p-ERK, JNK, and p-JNK (Cell Signaling Technology, Beverly, MA, USA). The quantity of lung samples required to extract the proteins with RIPA buffer (Enzyomic, Daejeon, Republic of Korea) was weighed and homogenized. The protein content was detected using the Bradford assay and the BSA standard after the supernatant was centrifuged at 13,200 rpm for 10 min at 4 $^{\circ}$ C (Bio-Rad Laboratories, Hercules, CA, USA), and the samples were placed into a pre-cast SDS polyacrylamide gel for 60–90 min at 100–130 V (constant voltage).

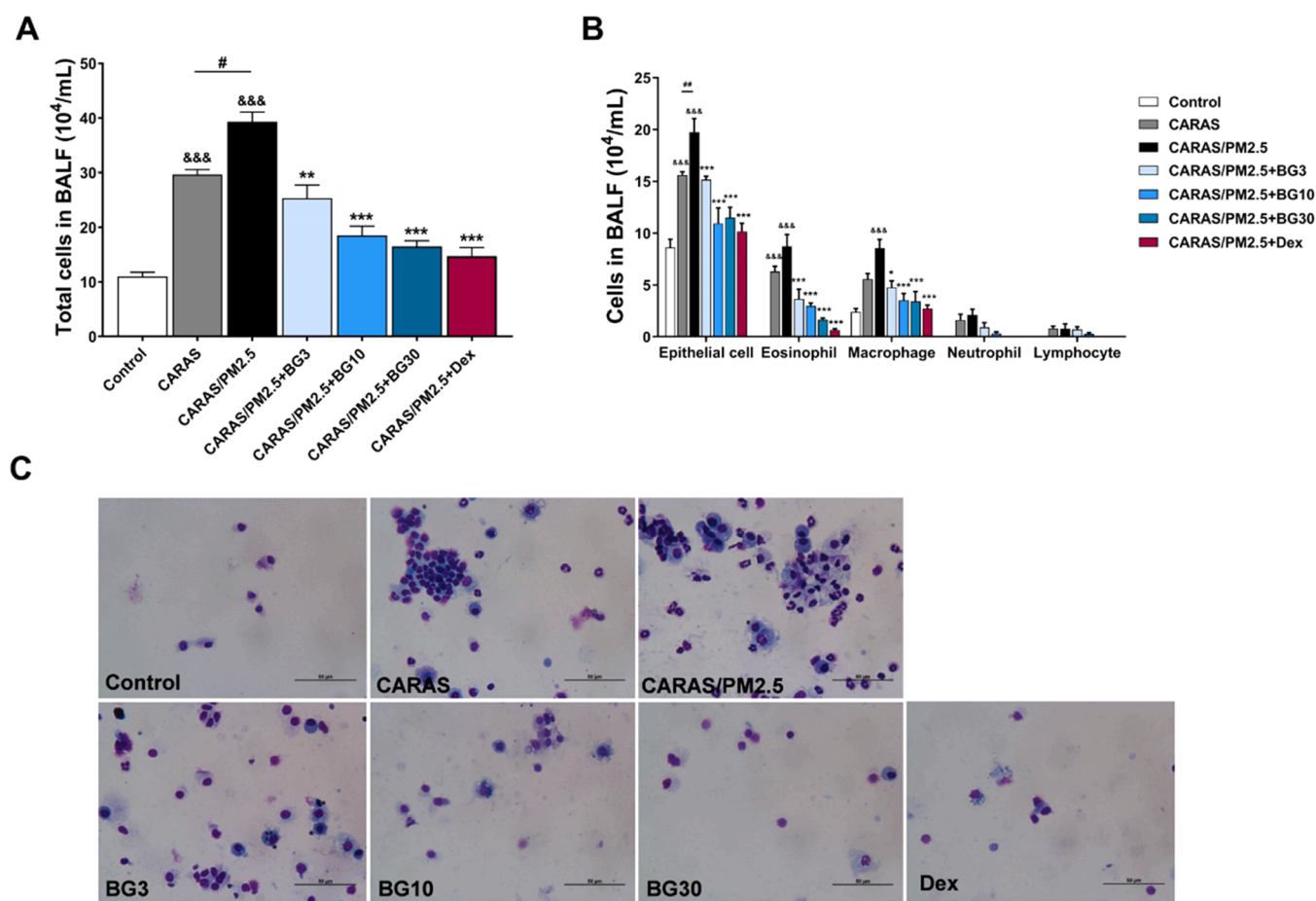


Fig. 4. Infiltration of total cells and differentiated inflammatory cells in BALF was reduced by bergapten. (A) Total cells. (B) Differentiated inflammatory cells. (C) Diff-Quik staining. Estimations of the quantities of both total and differentiated inflammatory cells by a hemocytometer. All data (n = 6 per group) are shown as mean $\pm$ SD. Significant deviations from the control group, $^{\&\&\&}$ $P < 0.001$, $^{\&\&}$ $P < 0.01$; from the CARAS group, $^{\#\#\#}$ $p < 0.001$; from the CARAS/PM2.5 group, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$. Scale bar = 50 μ m.

Afterward, as directed by the manufacturer, protein bands were transferred from the gel onto PVDF membranes (Bio-Rad Laboratories, Hercules, CA, USA) at 90 min of steady current transfer at 100 V in Tris-glycine transfer buffer (not to exceed 0.4 A). Following transfer, the membranes were rinsed briefly with Tris-buffered saline buffer with Tween 20 (TBST) and blocked in 5 % skim milk on a shaking platform for one hour. Then a specific primary antibody dissolved in TBST at the manufacturer's recommended concentration was incubated with the membranes overnight at 4 °C. After TBST washes, secondary antibodies were applied to the membranes, which were kept at ambient temperature for an additional hour. The detecting reagent (Thermo Fisher Scientific, Waltham, MA, USA) was used to calculate the density of bands, and ImageJ software was used to evaluate band intensity.

2.13. Statistical analysis

Prism software (v8.0, GraphPad, Boston, MA, USA) was used to analyze the entire data set, and the results are displayed as mean $\pm$ standard deviation (SD). Student's t-test, one-way analysis of variance, and Tukey's test were applied to analyze the data at a significance level of $P < 0.05$.

3. Results

3.1. Bergapten alleviated the nasal symptoms induced by OVA

Mice exposed to intranasal OVA challenge frequently exhibit signs of

nose rubbing and sneezing [36]. In this experiment, we determined how frequently those two symptoms occurred within 15 min of OVA challenge to examine the effects of bergapten. The CARAS and CARAS/PM2.5 groups displayed noticeably higher frequency than the control group, especially the CARAS/PM2.5 group. Oral bergapten in various doses effectively reduced the amount of rubbing and sneezing. The Dex group used as a positive control also showed significant inhibition. These results suggest that bergapten alleviates nasal symptoms in CARAS/PM2.5 mice, similar to those of CARAS (Fig. 2B, C).

3.2. Bergapten prevented inflammatory cells and total cells from infiltrating NALF and BALF

Next, we studied the anti-inflammatory effects of bergapten in the upper and lower respiratory tracts. To count all cells and the numbers of different inflammatory cells in NALF and BALF, we used a hemocytometer. Both the CARAS and CARAS/PM2.5 groups greatly outnumbered the control group in the total number of NALF and BALF cells, and the CARAS/PM2.5 group exceeded the CARAS group by a wide margin (Figs. 3A and 4A). However, the overall number of cells in NALF and BALF was dramatically and dose-dependently reduced by administration of bergapten, and Dex also had a strong inhibitory effect (Figs. 3A and 4A). Additionally, we discovered that the number of differentiated inflammatory cells (epithelial cells, eosinophils, and macrophages) in both NALF and BALF was considerably higher in the CARAS and CARAS/PM2.5 groups than in the control group, and there was a propensity for those cells to decrease in the bergapten and Dex groups

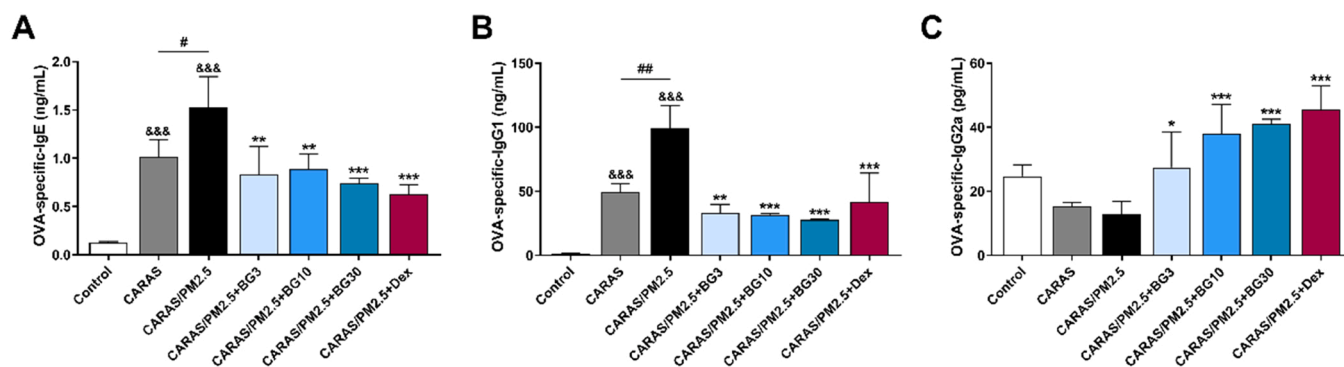


Fig. 5. Bergapten inhibited respiratory anaphylaxis by modulating the expression of OVA-specific antibodies in serum. (A) OVA-specific IgE, (B) OVA-specific IgG1, (C) OVA-specific IgG2a. In contrast to upregulation of OVA-specific IgG2a in the CARAS and CARAS/PM2.5 groups, treatment with bergapten or Dex substantially downregulated the production of OVA-specific IgE and IgG1. All data (n = 6 per group) are shown as mean $\pm$ SD. Significant deviations from the control group, &&& $p < 0.001$; from the CARAS group, ### $p < 0.001$, # $p < 0.05$; from the CARAS/PM2.5 group, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

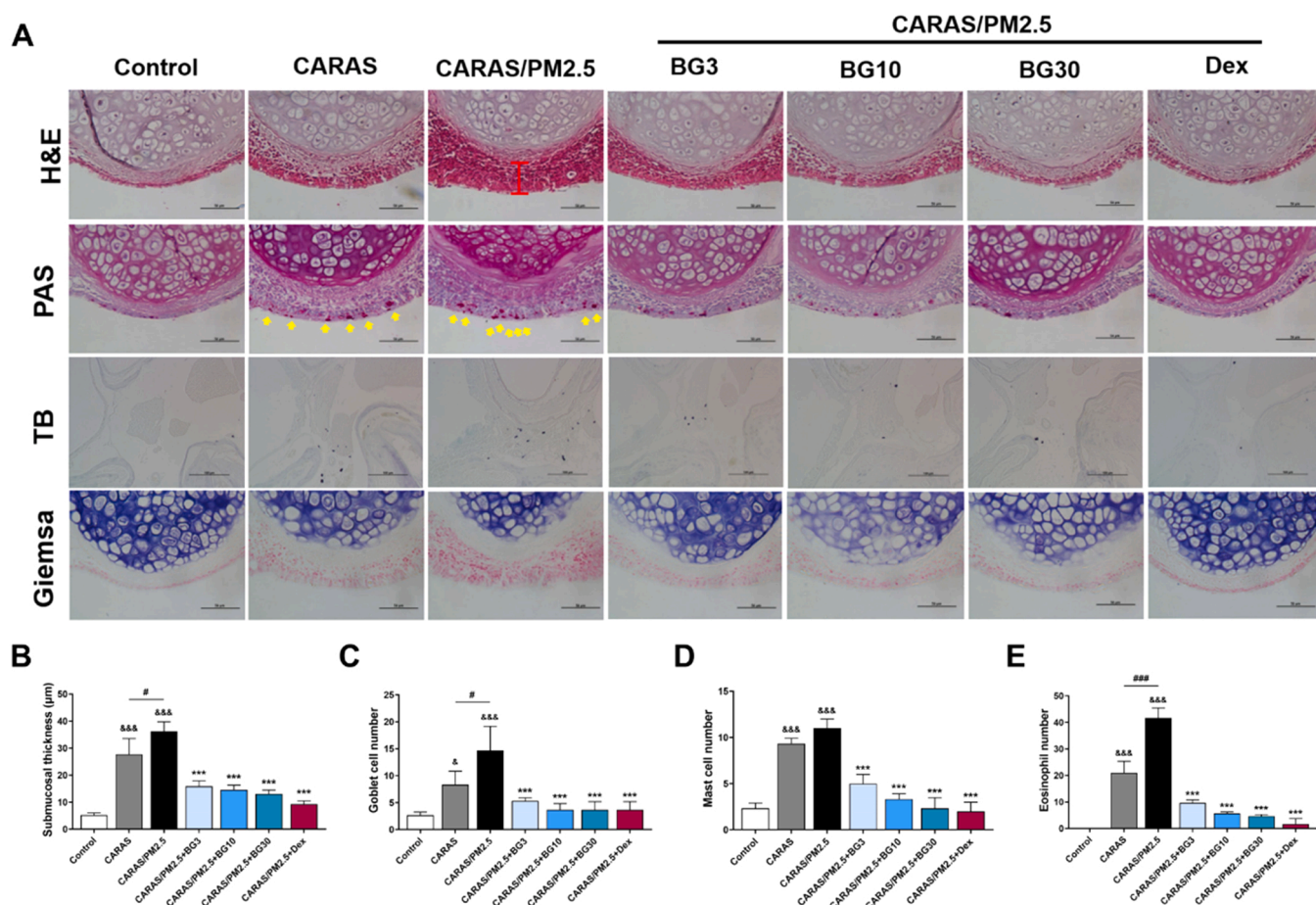


Fig. 6. Bergapten's effect on nasal histological alterations in CARAS/PM2.5 mice. (A) General morphology and epithelial thickness of the nasal mucosa visualized with H&E; Goblet cell hyperplasia (yellow arrow) illustrated with PAS; Mast cell infiltration (dark blue spots). TB; Infiltration of eosinophils shown with giemsa stain. (B) Submucosal thickness. (C) Quantity of goblet cells. (D) Quantity of mast cells. (E) Quantity of eosinophils infiltrated into the nasal epithelium. (n = 6 for each group) Total values are expressed as mean $\pm$ SD. Significant deviations from the control group, &&& $p < 0.001$, & $p < 0.05$; from the CARAS group, ### $p < 0.001$, # $p < 0.05$; from the CARAS/PM2.5 group, *** $p < 0.001$. Scale bar = 50, 100 μ m.

(Figs. 3B, C and 4B, C).

3.3. Bergapten controlled the expression of OVA-specific IgE, IgG1, and IgG2a in serum

To probe the immunomodulatory mechanism of bergapten in OVA-induced respiratory anaphylaxis, we examined the expression levels of

Th1-associated OVA-specific IgG2a and Th2-associated OVA-specific IgE and IgG1 in serum using ELISA. The results showed that Th2-related Ig level was significantly upregulated in OVA-sensitized mice, and the level in the CARAS/PM2.5 group was higher than that in the CARAS group. Furthermore, use of bergapten or Dex markedly suppressed OVA-specific IgE and IgG1 production (Fig. 5A, B). At the same time, the Th1-related IgG2a level tended to decrease in the CARAS and CARAS/

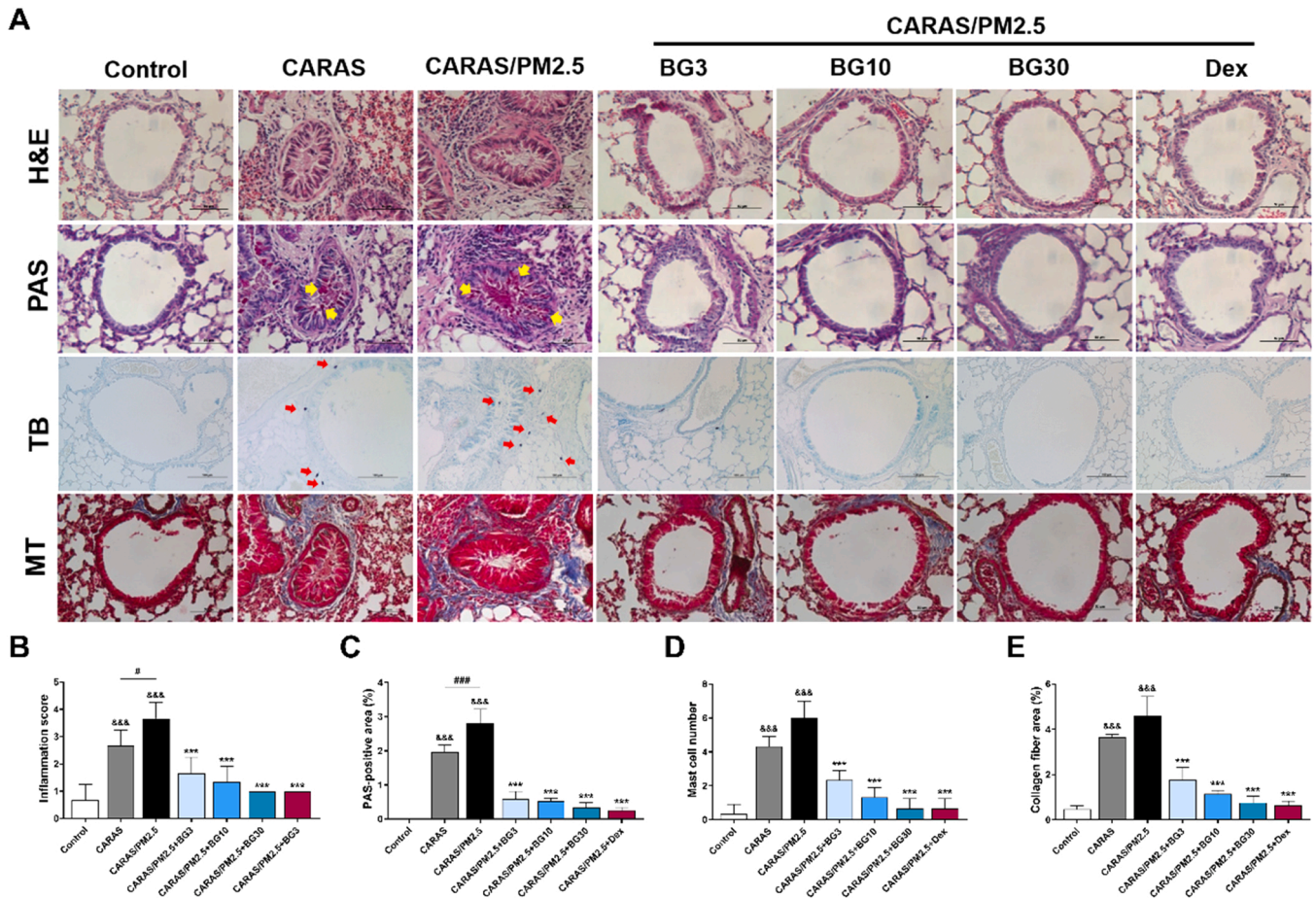


Fig. 7. Effects of bergapten on histopathological changes in lung tissues. (A) Histology of lung tissue visualized with H&E; Goblet cell hyperplasia (yellow arrows) illustrated with PAS; Mast cell immigration (red arrows) shown with TB; Collagen fiber deposition around the bronchioles and blood vessels after MT staining. (B) Lung inflammation score. (C) Number of goblet cells in the lung tissues. (D) Number of mast cells in the lung tissue. (E) Collagen fiber deposition. (n = 6 for each group) Each data point represents the mean $\pm$ SD. Significant deviation from the control group, &#&# p < 0.001; from the CARAS group, &#&# p < 0.001, # p < 0.05; from the CARAS/PM2.5 group, *** p < 0.001. Scale bar = 50, 100 μ m.

PM2.5 groups, and oral therapy with bergapten or Dex inhibited that reduction (Fig. 5C). Therefore, bergapten treatment could play a role in balancing Th1/Th2.

3.4. Bergapten prevented mast cell and eosinophil buildup, goblet cell hyperplasia, and thickening of the nasal mucosa

To determine the effects of bergapten on CARAS/PM2.5 mice, we examined histological changes in nasal mucosae. H&E staining showed that the sensitized mice had considerably thicker nasal mucosae than the control group, and the nasal mucosae of the mice treated with bergapten or Dex were thinner than those of the sensitized but untreated mice (Fig. 6A, B). Goblet cell hyperplasia was observably enhanced in the CARAS and CARAS/PM2.5 groups compared with the control group, and treatment with bergapten or Dex reduced this increase (Fig. 6A, C). TB staining of mast cells produced dark blue spots, as shown in Fig. 6. Compared with the control group, the CARAS and CARAS/PM2.5 groups showed mast cell infiltration in the nasal mucosa that was significantly decreased in the bergapten and Dex groups (Fig. 6A, D). Giemsa staining showed that administration of bergapten or Dex strongly reduced eosinophil aggregation, whereas eosinophils were overexpressed in the CARAS and CARAS/PM2.5 mice (Fig. 6A, E). All histological changes in the CARAS/PM2.5 group were much more substantial than those in the CARAS group. These findings suggest that bergapten therapy has some protective properties against penetration of inflammatory cells into the nasal mucosa.

3.5. Bergapten reduced lung tissue inflammation

Compared with the control group, both the thickness of the bronchial lumen and the level of inflammatory cell infiltration were considerably higher in the OVA-sensitized groups (Fig. 7A, B). Lung inflammation scores were evaluated according to thickness of the alveolar septum, edema, cell infiltration, hemorrhage, and alveolar distortion. Goblet cell hyperplasia and mucous hypersecretion were shown by PAS staining in the CARAS and CARAS/PM2.5 groups (Fig. 7A, C). The presence of mast cells inside the bronchioles was shown by TB staining (Fig. 7A, D). Moreover, significant collagen fiber deposition around the bronchioles and arteries was found in the CARAS and CARAS/PM2.5 groups by MT staining (Fig. 7A, E).

3.6. Bergapten regulated the expression of Th1- and Th2-associated cytokines in NALF and BALF

Cytokine levels in NALF and BALF were evaluated by ELISA to ascertain bergapten's regulatory effects on the Th1 and Th2 immune response. Notably, in contrast to the healthy control group, IL-4, IL-5, and IL-13, which relate to Th2, were considerably upregulated in the sensitized groups, and cytokines associated with Th1 (IL-12 and IFN- γ) were suppressed (Fig. 8A–J). Oral administration of bergapten or Dex inhibited Th2 cytokine upregulation and Th1 cytokine downregulation compared with the CARAS and CARAS/PM2.5 groups (Fig. 8A–J).

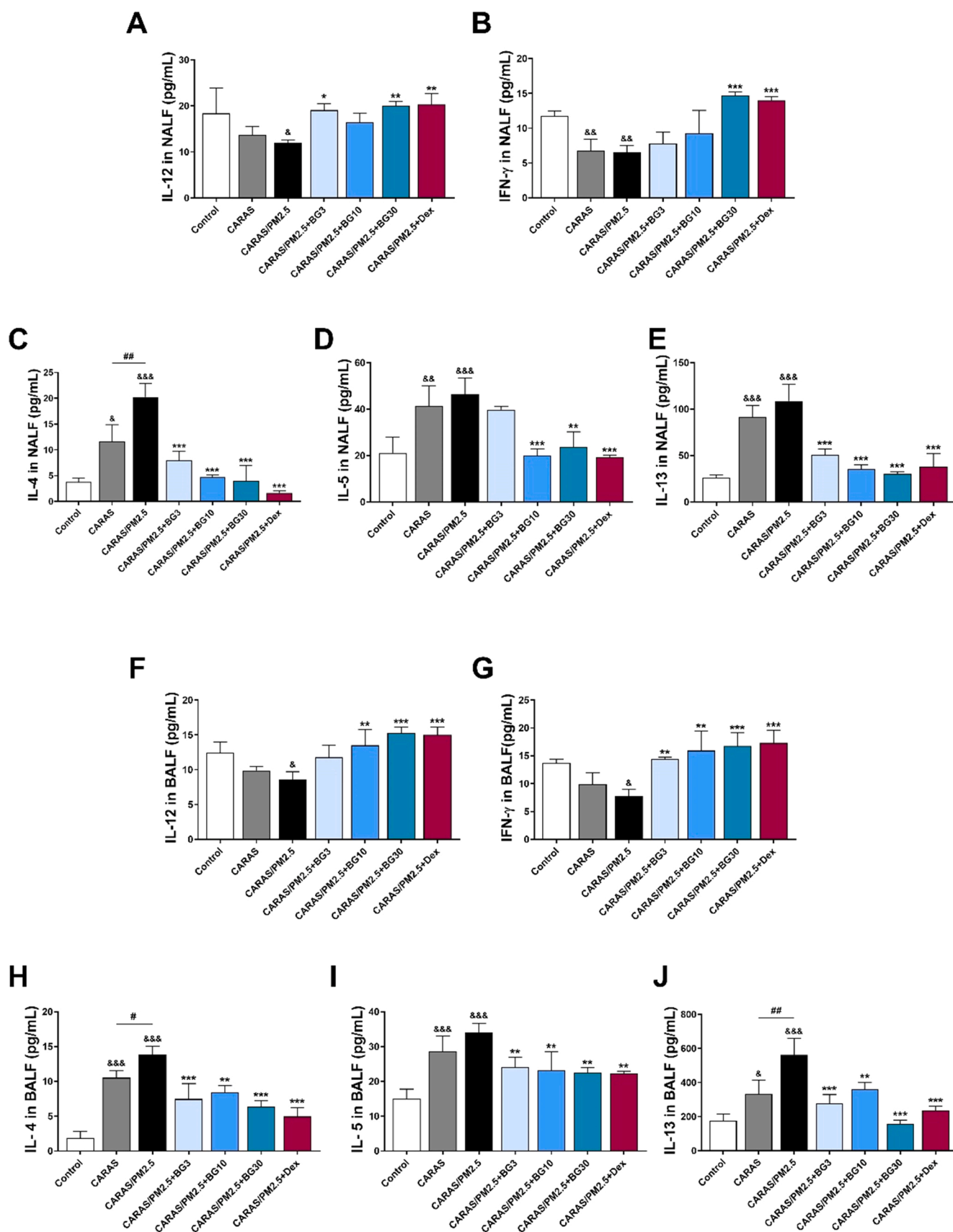


Fig. 8. Bergapten regulated the expression of Th1- and Th2-associated cytokines in NALF and BALF from CARAS/PM2.5 mice. The expression of Th1-associated cytokines (A) IL-12 and (B) IFN-γ and Th2-associated cytokines (C) IL-4, (D) IL-5, and (E) IL-13 in NALF and BALF (F–J). (n = 6 for each group) The total values are shown as mean ± SD. Significant deviations from the control group, &&& $P < 0.001$, && $P < 0.01$, & $P < 0.05$; from the CARAS group, ## $p < 0.01$, # $P < 0.05$; from the CARAS/PM2.5 group, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

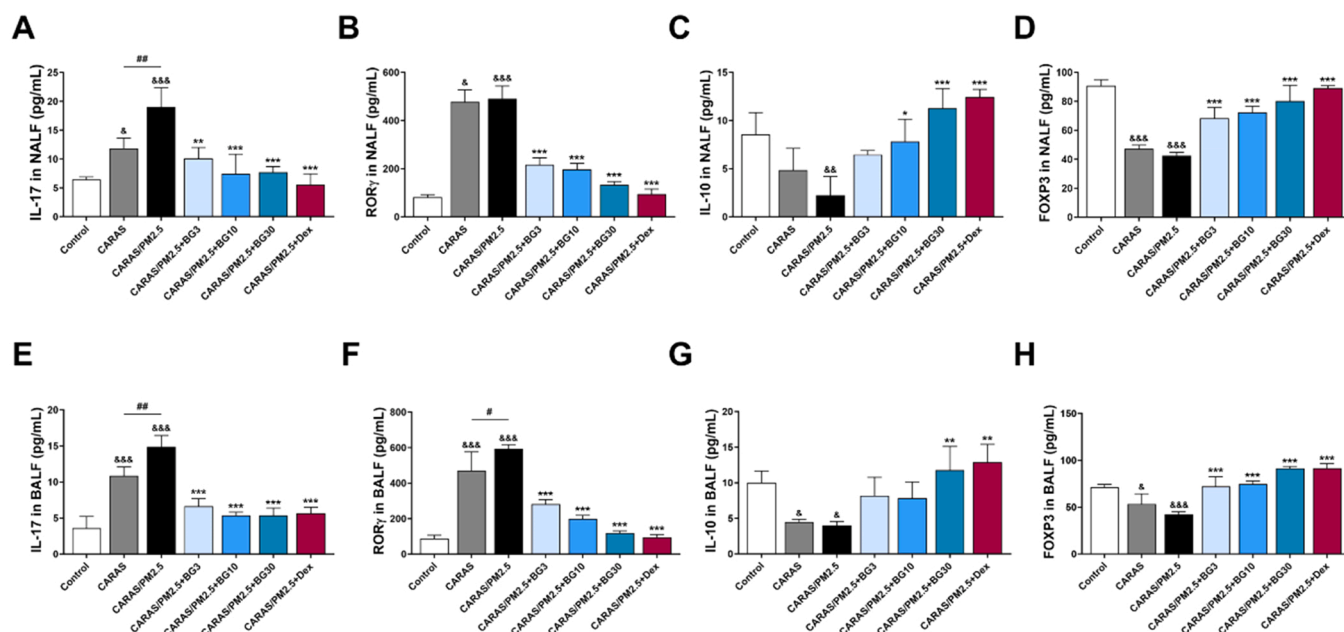


Fig. 9. Effect of bergapten on Th17- and Treg-associated cytokines and transcription factors in the NALF and BALF of CARAS/PM2.5 mice. Th17 cytokine (A) IL-17, transcription factor (B) ROR γ , Treg cytokine (C) IL-10, and transcription factor (D) FOXP3 expression levels were identified in NALF and BALF (E–H) using ELISA. All data (n = 6 per group) are shown as mean $\pm$ SD. Significant deviations from the control group, &&& p < 0.001, && p < 0.01, & p < 0.05; from the CARAS group, ## p < 0.01, # p < 0.05; from the CARAS/PM2.5 group, *** p < 0.001, ** p < 0.01, * p < 0.05.

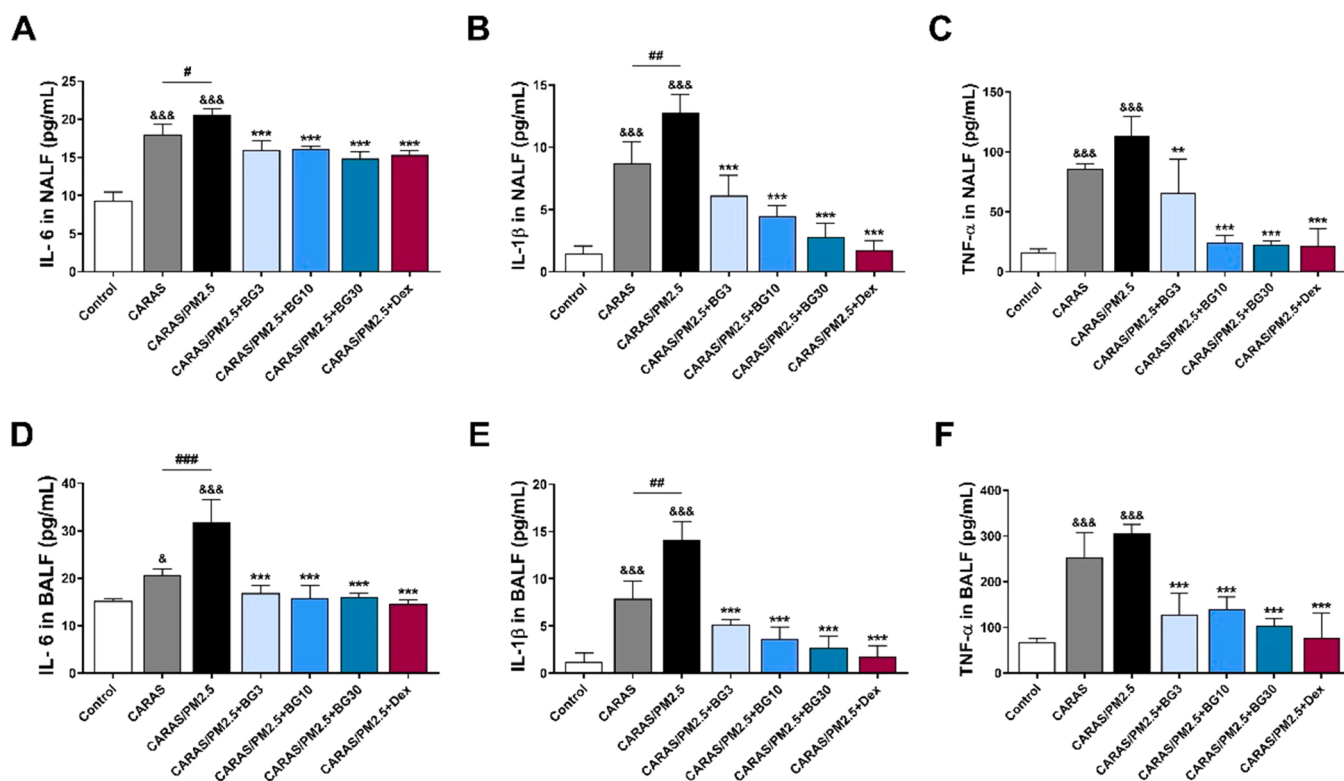


Fig. 10. Bergapten prevented the release of inflammatory cytokines into NALF and BALF. The expression of pro-inflammatory cytokines (A, D) IL-6, (B, E) IL-1 β , and (C, F) TNF- α was calculated and analyzed using ELISA. All data are shown as mean $\pm$ SD (n = 6 per group). Significant deviations from the control group, &&& p < 0.001, & p < 0.05; from the CARAS group, ### p < 0.001, ## p < 0.01, # p < 0.05; from the CARAS/PM2.5 group, *** p < 0.001.

3.7. Bergapten modulated the expression of Th17 and Treg cytokines and transcription factors in NALF and BALF

To ascertain whether bergapten contributed to the Th17/Treg

imbalance in CARAS/PM2.5 mice, cytokines and transcription factors associated with Th17 cells (IL-17 and ROR γ) and Treg (IL-10 and FOXP3) were measured using ELISA. IL-17 and ROR γ levels in the CARAS and CARAS/PM2.5 groups were noticeably higher than in the

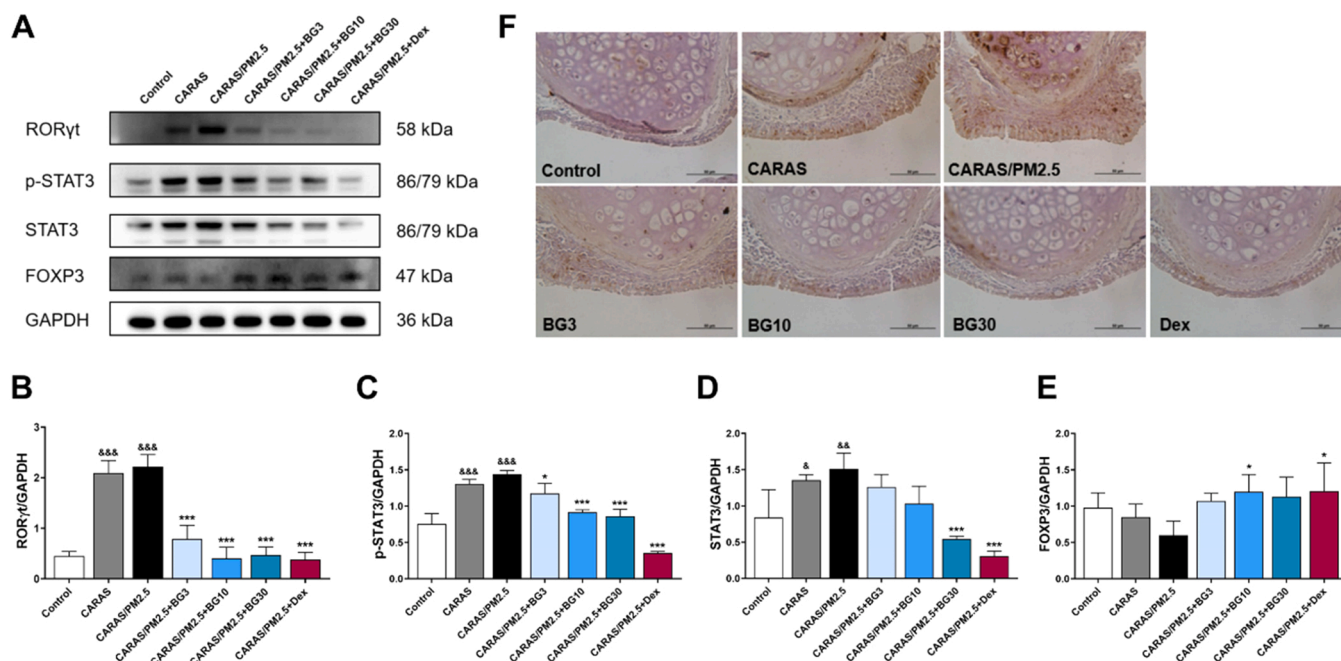


Fig. 11. Bergapten inhibited the STAT3 signaling pathway and upregulated FOXP3 level. (A) The expression of STAT3 signaling-associated proteins (RORγt, p-STAT3, and STAT3) and a Treg-related protein (FOXP3) in lung tissue was determined through western blotting. (B) RORγt/GAPDH, (C) p-STAT3/GAPDH, (D) STAT3/GAPDH, and (E) FOXP3/GAPDH were calculated in ImageJ. (F) Immunohistochemistry of p-STAT3 in nasal tissue. All data (n = 6 per group) are shown as mean ± SD. Significant deviations from the control group, ^{***} P < 0.001, ^{**} P < 0.01, ^{*} P < 0.05; from the CARAS/PM2.5 group, ^{***} P < 0.001, ^{*} P < 0.05. Scale bar = 50 μm.

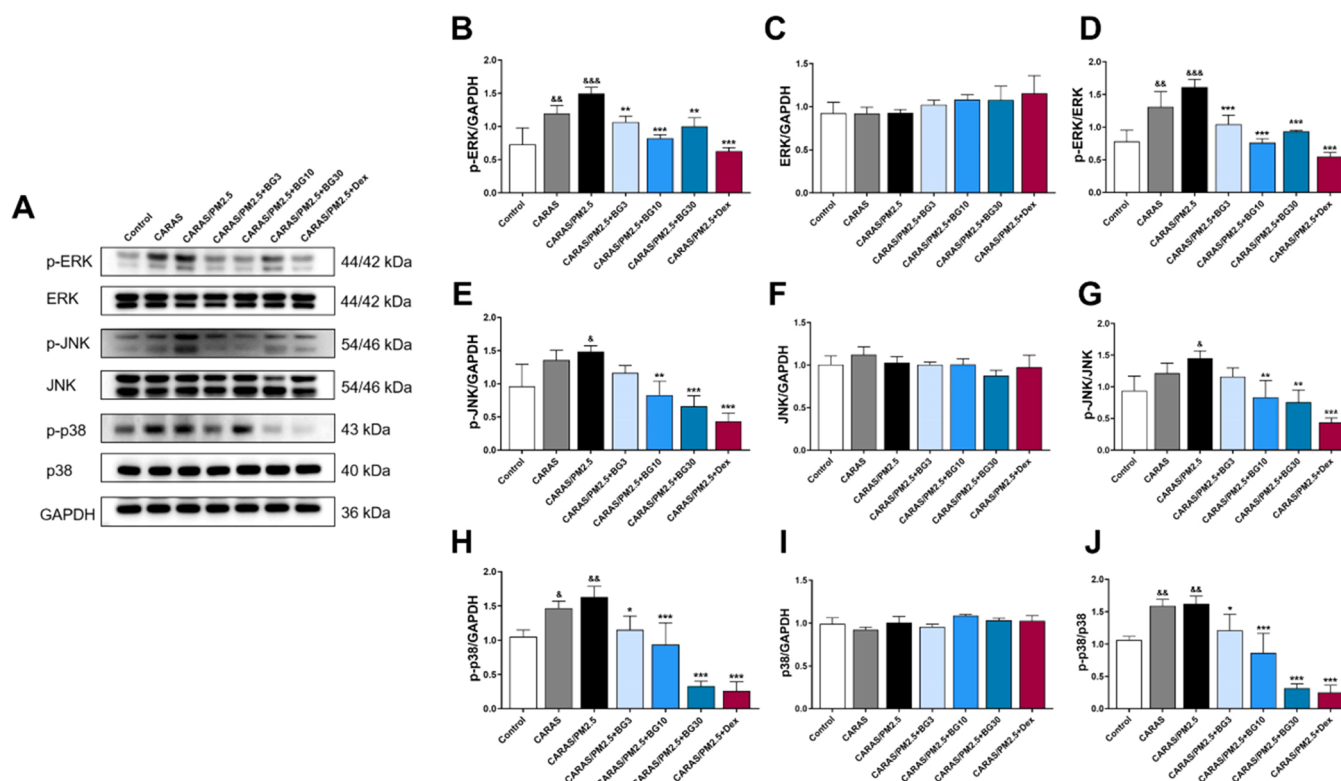


Fig. 12. Bergapten inhibited the MAPK signaling pathway in CARAS/PM2.5 mice. (A) Expression of MAPK signaling pathway-associated proteins (ERK, p-ERK, JNK, p-JNK, p38, p-p38) in lung tissue was quantified via western blotting. (B) p-ERK/GAPDH, (C) ERK/GAPDH, (D) p-ERK/ERK, (E) p-JNK/GAPDH, (F) JNK/GAPDH, (G) p-JNK/JNK, (H) p-p38/GAPDH, (I) p38/GAPDH, and (J) p-p38/p38 ratios were calculated using ImageJ. All data (n = 6 per group) are shown as mean ± SD. Significant deviations from the control group, ^{***} P < 0.001, ^{**} P < 0.01, ^{*} P < 0.05; from the CARAS/PM2.5 group, ^{***} P < 0.001, ^{**} P < 0.01, ^{*} P < 0.05.

normal control group, and that increase was significantly reversed by bergapten or Dex therapy, which also significantly reduced the secretion of the mediators (Fig. 9A, B and E, F). In the CARAS and CARAS/PM2.5 groups, IL-10 and FOXP3 levels were substantially reduced in NALF and BALF, respectively, and those levels increased when bergapten or Dex was administered (Fig. 9C, D and G, H).

3.8. Bergapten inhibited the expression of pro-inflammatory cytokines in NALF and BALF

To further elucidate the antiallergic mechanism of bergapten in CARAS/PM2.5 mice, we measured the amounts of TNF- α , IL-6, and IL-1 β cytokines, which are primarily pro-inflammatory, in NALF and BALF. The release of TNF- α , IL-6, and IL-1 β in NALF and BALF was noticeably higher in the sensitized groups than the control group. However, their expression in NALF and BALF was considerably lower in the groups receiving bergapten or Dex compared with the CARAS and CARAS/PM2.5 groups (Fig. 10A–F). Therefore, we conclude that bergapten works to alleviate allergy symptoms in CARAS/PM2.5 mice by suppressing the release of pro-inflammatory cytokines.

3.9. Bergapten inhibited the STAT3 signaling pathway and upregulated FOXP3 level

To further probe the mechanisms of bergapten in CARAS/PM2.5 mice, we detected the expression of the Th17-related STAT3 signaling pathway using ROR γ t, p-STAT3, and STAT3 levels and the expression of the Treg-related transcription factor FOXP3. A western blot examination of the CARAS and CARAS/PM2.5 groups showed that the protein expression of ROR γ t, p-STAT3, and STAT3 was much higher than in the control group (Fig. 11A–D), indicating that the CARAS/PM2.5 model induced by OVA presented an activated STAT3 signaling pathway. Bergapten or Dex therapy effectively diminished ROR γ t, p-STAT3, and STAT3 protein expression (Fig. 11A–D). In contrast, treatment with bergapten or Dex boosted the expression of FOXP3, which was lower in the CARAS and CARAS/PM2.5 groups (Fig. 11A, E). To further confirm the inhibitory effect of bergapten in the CARAS/PM2.5 mouse model, p-STAT3 expression in nasal tissues was detected by IHC. We found that p-STAT3 expression was upregulated in the CARAS and CARAS/PM2.5 groups and was higher in the CARAS/PM2.5 group than the CARAS group. Furthermore, bergapten or Dex treatment greatly reduced p-STAT3 expression (Fig. 11F).

3.10. Bergapten inhibited the MAPK signaling pathway in CARAS/PM2.5 mice

Next, we explored the inhibitory effects of bergapten on activation of the MAPK signaling pathway. We used western blotting to investigate protein expression along the MAPK signaling pathway, which is directly associated with the incidence and progression of the inflammatory response. ERK, JNK, and p38 phosphorylation was strong in the lung tissue of the CARAS/PM2.5 group compared with the control group (Fig. 12A) and was considerably reduced by bergapten (Fig. 12B, E, H). Additionally, the p-ERK/ERK, p-JNK/JNK, and p-p38/p38 ratios in the bergapten therapy groups all displayed significant declining trends (Fig. 12D, G, J). However, ERK, JNK, and p38 expression did not differ among the groups (Fig. 12C, F, I).

4. Discussion

AR is characterized by persistent upper respiratory tract inflammation, whereas asthma is characterized by chronic lower respiratory tract inflammation. They are similar in their pathogenesis and local pathological changes. CARAS often presents as persistent allergic inflammatory responses in patients' upper and lower respiratory tracts [5,6]. As industrialization spreads worldwide, air pollution is becoming

progressively worse. PM2.5 has emerged as the foremost environmental risk factor that poses a significant threat to human health [21]. Asthma, pneumonia, pulmonary dysfunction, lung cancer, and other respiratory system injuries can be caused by inhaling PM2.5 because it can reach the terminal bronchioles and enter the alveoli [20].

A furanocoumarin called bergapten (5-methoxysporalen) is extracted from bergamot essential oil. Earlier research has shown that bergapten has notable antioxidant, anticancer, and anti-inflammatory properties [24–26]. Bergapten can inhibit the colitis model induced by acetic acid, lipopolysaccharide-induced hypersensitivity, and ethanol-induced ulcer [28–31,37]. Therefore, in this study, we conducted an in-depth exploration on the anti-inflammatory effects of bergapten. We started by creating a murine CARAS/PM2.5 model in BALB/c mice, and then we examined how bergapten administration modified the symptoms and immunomodulatory processes of the sensitized mice. Specifically, we assessed nasal symptoms, changes in inflammatory cell infiltration in the nasal mucosae and lung tissues, and the Th1–Th2 and Treg–Th17 immune balance. We also analyzed any anti-inflammatory benefits of bergapten on the STAT3 and MAPK signaling pathways, which are involved in the onset of inflammation.

Consistent with previous investigations, we found that mice exposed to OVA had clear nasal symptoms of scratching and sneezing [36,38]. Our nasal histological investigation revealed swollen mucosae with increased inflammatory cell infiltration, goblet cell hyperplasia, mast cells, and eosinophil recruitment in the CARAS and CARAS/PM2.5 groups compared with the control group. Lung histology showed allergic alterations in the lower respiratory tract as well, including inflammatory cell infiltration, goblet cell proliferation, mast cell recruitment, and collagen fiber deposition. Meanwhile, innate immune system activation produces a series of reactions, such as elevation in eosinophils, macrophages, and neutrophils in NALF and BALF. Additionally, we found an increase in serum OVA-specific IgE expression and the levels of cytokines associated with Th2 (IL-4, IL-5, and IL-13) NALF and BALF, whereas the expression of cytokines associated with Th1 (IL-12 and IFN- γ) was markedly suppressed. Our data indicate that the CARAS/PM2.5 group had more severe symptoms than the CARAS group. Those outcomes demonstrate our successful CARAS/PM2.5 mouse model.

Allergic inflammation is a class of inflammatory disease dominated by type 2 immune responses and showing a trend of increasing Th2 cells. IL-4, IL-5, and IL-13 are Th2-related cytokines that are secreted more frequently in allergic inflammation than in other inflammatory diseases. The increased secretion of Th2-related cytokines prompts B cells to create IgE, which results in infiltration of various inflammatory cells [7, 39]. Specific IgE antibodies have been shown to mediate mast cell degranulation by attaching to the Fc ϵ R1 receptor on the surfaces of mast cells [40]. This generates inflammatory mediators and the development of early allergic symptoms, including rubbing and sneezing [41,42]. Our findings demonstrate that bergapten therapy considerably decreased those initial allergy symptoms (rubbing and sneezing). The airway lumen of patients with late anaphylaxis frequently contains high concentrations of inflammatory cells such as eosinophils and neutrophils, which can damage and inflame the airway epithelium. To continue our investigation into the effects of bergapten on CARAS/PM2.5 mice, we methodically examined histological alterations in nasal mucosae and lung tissues, as well as the expression of different cytokines in NALF and BALF. The total number of cells and the numbers of differentiated inflammatory cells (eosinophils, neutrophils, and macrophages) in NALF and BALF were noticeably lower in the bergapten-treated groups than the CARAS and CARAS/PM2.5 groups. The histological data suggest that bergapten effectively lessened inflammation in the nasal mucosa and lung tissue brought on by OVA. Bergapten therapy also significantly improved nasal septum epithelial thickness and reduced mast cell and eosinophil infiltration and goblet cell proliferation. Furthermore, bergapten lessened peribronchial inflammation, mast cell aggregation, goblet cell hyperplasia, and peribronchial collagen fiber deposition in

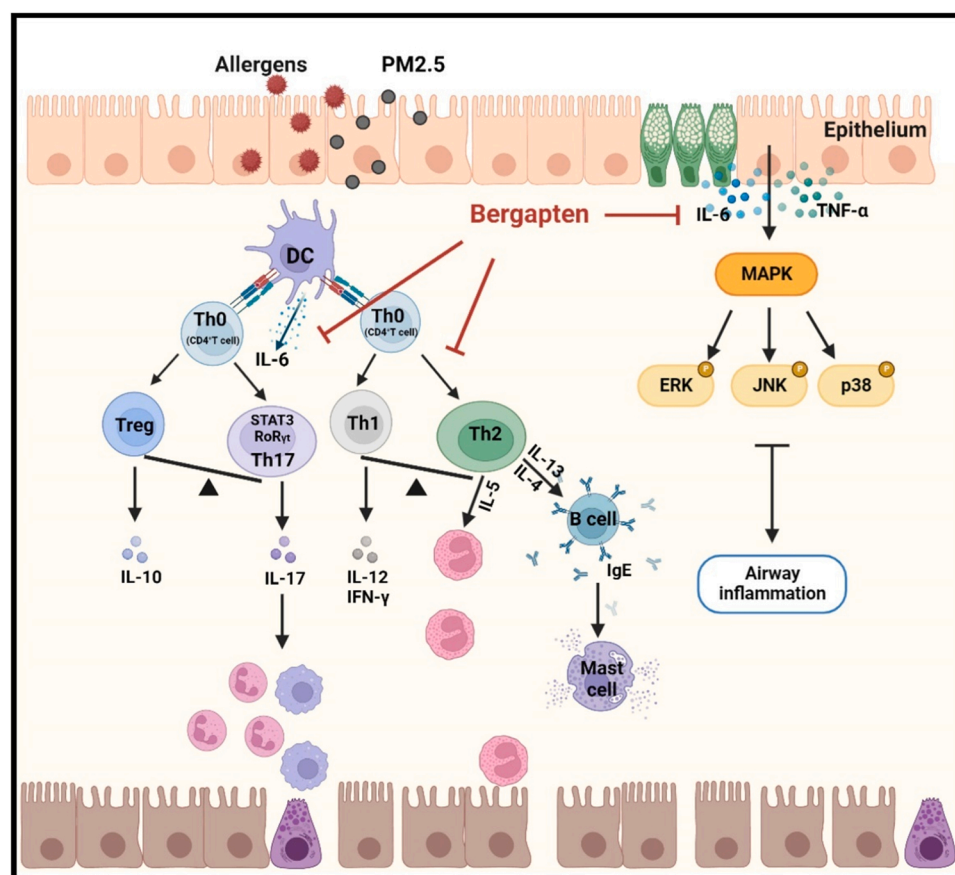


Fig. 13. The mechanism of action for bergapten in CARAS/PM2.5 mice. Th0 cells are encouraged to develop into Th2 and Th17 cells by exposure to exogenous allergens. Th2 cells that have been triggered by an antigen activate B cells to produce specific IgE antibodies that engage with mast cells, causing them to degranulate and release inflammatory cytokines and chemokines. Both IgE antibodies and mast cells contribute to the development of allergic respiratory inflammation, along with the cytokines generated by Th2 cells. Another important factor in the inflammatory response is an imbalance between Th17 cells and Treg. Activation of the MAPK signaling pathway speeds the inflammatory process. In this instance, bergapten reduced the inflammation by balancing the Th1/Th2 and Th17/Treg ratios, as well as by blocking the MAPK signaling pathway.

lung tissues. Thus, bergapten might be used to treat allergic inflammatory reactions in the upper and lower respiratory tracts.

According to earlier studies, Th1/Th2 balance is a key process for preserving an individual's immune function. When exposed to allergens that trigger allergic inflammation, the Th2 immune response is initiated, which elevates the secretion of IL-4, IL-5, and IL-13 [43]. IL-5 promotes the recruitment of eosinophils [44], and IL-4 and IL-13 stimulate B cells to start producing specific IgE antibodies [45,46], which interact with specific receptors on mast cell surfaces to promote mast cell degranulation and release allergenic mediators, resulting in increase in the release of pro-inflammatory cytokines (IL-1 β and IL-6) and chemokines [41]. In this investigation, we discovered that bergapten therapy significantly downregulated Th2-related Ig level and upregulated Th1-related Ig level. The use of bergapten treatment also lowered IL-4, IL-5, and IL-13 levels in NALF and BALF. In parallel, the bergapten-treated group showed increased expression of IL-12 and IFN- γ in NALF and BALF. These results indicate that the Th1/Th2 imbalance associated with allergic reaction is relieved by bergapten therapy.

Moreover, Treg and Th17 cells appear to be necessary for the pathogenic process of allergic airway inflammatory disorders, indicating a Treg/Th17 imbalance as important cause of autoimmune inflammatory illnesses [47]. In that process, various pro-inflammatory factors (IL-6, IL-1 β , and TNF- α) and the STAT3 signaling pathway are strongly associated with the development of Th17 cells and Treg [12,48]. Under the combined influence of IL-6 and IL-1 β , CD4⁺T cells develop into Th17 cells, which release IL-17, draw in neutrophils, and increase inflammation at the site of infection. On the other hand, after being triggered by homologous antigens, Treg exert a negative immunomodulatory effect by secreting anti-inflammatory cytokines such as IL-10, restricting the activity of different immune cells and ultimately reducing the immune response. Furthermore, when IL-6 and IL-1 β work together to regulate Th17 cell development, the action of STAT3 phosphorylation can be

amplified and sustained, which increases the production of ROR γ and further encourages Th17 differentiation [49–52]. Therefore, we examined IL-6, IL-1 β , and TNF- α expression in this study. The bergapten-treated groups had lower levels of those cytokines in NALF and BALF than the CARAS and CARAS/PM2.5 groups. Furthermore, bergapten administration enhanced IL-10 and FOXP3 levels in NALF and BALF and noticeably decreased IL-17 and ROR γ levels compared with the CARAS and CARAS/PM2.5 groups. Western blotting demonstrated that bergapten treatment notably downregulated the expression of STAT3 signal-related proteins (ROR γ t, p-STAT3, and STAT3). Conversely, expression of the Treg-related protein FOXP3 increased in the treated groups. According to our immunohistochemical analyses, p-STAT3 expression was noticeably downregulated in the nasal tissues of mice given bergapten. Therefore, it is possible that bergapten affected CARAS/PM2.5 mice by controlling the Th17/Treg imbalance.

The MAPK pathway is crucial for the incidence and progression of respiratory inflammation and is involved in immunological dysregulation [53–55]. TNF- α and other inflammatory mediators can promote the phosphorylation of MAPK proteins such as ERK, JNK, and p38 to activate immune cells and inflammatory response transcription factors, which encourage the production of inflammatory cytokines and chemokines and hasten the inflammatory process [56]. As previously documented in the literature, GATA-3 has a key role in allergic disorders by controlling the release of cytokines from Th2 cells. Nuclear translocation of GATA-3 is dependent on the p38 MAPK's phosphorylation of its serine residues, suggesting that p38 MAPK is essential for modulation of the Th2 cytokine genes and that inhibiting p38 MAPK may be beneficial for treating allergic inflammation [55]. Th polarization is influenced by mast cells, and mast cell degranulation results in additional Th2 and fewer Th1 responses [40]. Bergapten has previously demonstrated anti-allergic effects and mast cell stabilization [37]. Therefore, by examining the expression of important proteins connected to the

MAPK signaling route in the lung tissue of each group of mice, we confirmed the role of bergapten in the MAPK signaling pathway in this study. In our western blot examination, bergapten treatment clearly lowered the phosphorylation of ERK, JNK, and p38. Moreover, bergapten treatment decreased the p-ERK/ERK, p-JNK/JNK, and p-p38/p38 ratios. These results indicate that downregulation of the MAPK signaling pathway might be a potential mechanism by which bergapten reduced pulmonary inflammation in CARAS/PM2.5 mice (Fig. 13). Our study has limitations, such as individual variations. To validate the therapeutic potential of bergapten to decrease respiratory inflammation, more research will be required.

5. Conclusion

Our investigation showed that bergapten administration can limit the expression of serum OVA-specific IgE and IgG1 and proinflammatory cytokines and can minimize the infiltration of different inflammatory cells and collagen fiber deposition in nasal mucosa and lung tissue. Bergapten can also control the Th1/Th2 and Treg/Th17 ratios by suppressing the expression of Th2 and Th17 cytokines and functions as a preventative and therapeutic agent by modifying the MAPK signaling pathway. These findings collectively suggest that bergapten has substantial anti-inflammatory and antiallergenic properties and tremendous potential for treating allergic airway disorders. They offer an additional option for future treatment of associated disorders.

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CRediT authorship contribution statement

Yuna Jiang: Data curation, Investigation, Writing - original draft, Writing and Revising-original draft. **Thi Van Nguyen:** Data curation. **Juan Jin:** Investigation. **Zhen Nan Yu:** Resources. **Chang Ho Song:** Supervision, Visualization. **Ok Hee Chai:** Supervision, Project administration, Writing - review & editing, Revising. The final version of the manuscript was read and approved by all the authors.

Declaration of Competing Interest

The authors affirm that this paper is free of any conflicts of interest.

Data Availability

Data will be made available on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.biopha.2023.114959](https://doi.org/10.1016/j.biopha.2023.114959).

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