

ORIGINAL ARTICLE

Experimental Models of Allergic Disease

Tetrahydrocurcumin, a major metabolite of curcumin, ameliorates allergic airway inflammation by attenuating Th2 response and suppressing the IL-4R α -Jak1-STAT6 and Jagged1/Jagged2 -Notch1/Notch2 pathways in asthmatic mice

Bin Lin Chen^{1,2} | Yan Qiu Chen³ | Bai Hui Ma^{1,2} | Si Fei Yu⁴ | Li Yue Li^{5,6} |
 Qing Xiang Zeng³ | Yu Tao Zhou^{5,6} | Yin Fan Wu^{1,2} | Wen long Liu³  |
 Jian Bo Wan⁷ | Yan Yang^{1,2} | Chun Wei Li^{5,6} 

¹School of Public Health (Shenzhen), Sun Yat-sen University, Guangzhou, China

²Guangdong Provincial Key Laboratory of Food, Nutrition and Health, School of Public Health, Sun Yat-sen University (Guangzhou Campus), Guangzhou, China

³Department of Otolaryngology, Guangzhou Women and Children Medical Centre, Guangzhou, China

⁴Institute of Immunology, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou, China

⁵Department of Otolaryngology, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, China

⁶Guangzhou Key Laboratory of Otorhinolaryngology, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, China

⁷State Key Laboratory of Quality Research in Chinese Medicine, Institute of Chinese Medical Sciences, University of Macau, Macau, China

Correspondence

Chun Wei Li, Department of Otorhinolaryngology, The First Affiliated Hospital, Sun Yat-Sen University, Guangzhou, China.

Email: hi_chunwei@aliyun.com

Yan Yang, School of Public Health (Shenzhen), Sun Yat-sen University (Shenzhen Campus), Guangzhou, China. Email: yangyan3@mail.sysu.edu.cn

Funding information

Fundamental Research Funds for the Central Universities, Grant/Award Number:

Summary

Background: Curcumin (Cur), derived from *Curcuma* species, exhibits anti-inflammatory, antioxidant, and anticancer effects. Although Cur has some beneficial effects on asthma, its clinical application is limited by its low bioavailability. Tetrahydrocurcumin (THC), the major active metabolite of Cur, has multiple biological functions, similarly to Cur, and importantly, it showed enhanced bioavailability in tissues and plasma. However, the effect of THC on asthma has not been reported.

Objective: The current study sought to investigate the efficacy of dietary THC on allergic asthma compared to that of Cur in an animal model.

Methods: The anti-inflammatory effects of Cur and THC were evaluated in an ovalbumin-induced asthmatic mouse model. The nasal symptoms, pathological alterations of the lung tissues, oxidants and antioxidants, cytokine production, T cell subsets, and Th2-related signalling pathway activity were assessed.

Results: Both THC and Cur had beneficial effects on asthmatic mice with regard to nasal symptoms, pathological changes (eosinophils and mucus hyper-production), oxidative stress (malondialdehyde), cytokine production (IL-13), Th17 and cytotoxic T cell subsets, and Th2 signalling pathway (IL-4R α -Jak1-STAT6 and Jagged1/Jagged2-Notch1/Notch2 axis) activity. THC was more effective than Cur in suppressing tissue eosinophilia, mucus production, and IL-4R α /Jak1/STAT6 pathway activity. Furthermore, only THC inhibited peripheral eosinophil levels, Th2 cytokines (IL-4 and IL-5), and Th2 cell subsets and enhanced an antioxidant enzyme (glutathione).

Conclusion and Clinical Relevance: The above results demonstrated for the first time that THC was superior to Cur in modulating allergic asthmatic phenotypes, especially attenuating the Th2 response. THC might be a potentially effective agent for asthma treatment.

17ykzd19; Guangzhou Science and Technology Programme, Grant/Award Number: 201605030003; Natural Science Foundation of Guangdong Province, Grant/Award Number: 2015A050502020, 2017A030313621; National Natural Science Foundation of China, Grant/Award Number: 81500772, 81700892, 81770983; Guangdong Provincial Medical Science and Technology Research Fund, Grant/Award Number: B2016005

KEYWORDS

airway inflammation, asthma, dietary factor, tetrahydrocurcumin, Th2 response

1 | INTRODUCTION

Allergic asthma is a chronic airway inflammatory disease that is pathologically characterized by an increase in inflammatory cells (especially eosinophils), aberrant airway remodelling (goblet cell metaplasia and smooth muscle cell hyperplasia), and elevation of multiple pro-inflammatory cytokines (type 2 helper T cell [Th] dominant response). The incidence of allergic asthma has strongly increased over the past three decades, both in developed and developing countries, concomitant with the rise in the adoption of the “western lifestyle”. Dietary factors play important roles in the development of allergic diseases, but they also have beneficial effects in modulating immune homeostasis and response.^{1,2} High-fat diet-induced obesity has been confirmed to induce or exacerbate asthma symptoms, possibly by dysregulation of white adipose tissue immunity, increased adipokine release from the visceral and subcutaneous fat tissue, and abnormal regulation of T cell metabolism via the mammalian target of rapamycin complex 1 (mTORC1) pathway.³ In contrast, some dietary intervention approaches may help prevent or alleviate allergic diseases by suppressing Th2 and/or Th17 responses and enhancing Treg activities, and these dietary factors include polyunsaturated fatty acids (eg, omega-3, docosahexaenoic acid),^{4,5} dietary fibres,⁶ short-chain fatty acids,⁷ vitamin A,⁸ and vitamin D3.⁹

Curcumin (Cur), a yellow pigment present in the Indian spice turmeric, is a nutraceutical that exhibits a variety of biological activities, including anti-inflammatory, anticancer, antioxidant, wound healing, and antimicrobial effects.¹⁰ The effect of Cur on asthma has been reported in human studies, which showed that administration of Cur capsules improved mean forced expiratory volume in 1 s values and airway obstruction.¹¹ The mechanism underlying Cur modulation of allergic reactions could be suppression of Syk kinase-dependent signalling events in mast cells,¹² inhibition of the NF- κ B pathway, suppression of Th17 cells,¹³ inhibition of lymphocyte proliferation,¹⁴ reduction of NO/inducible nitric oxide synthase,¹⁵ inhibition of the recruitment of eosinophils,¹⁶ and down-regulation of the Notch pathway in peripheral T cell proliferation.¹⁷

Although Cur has pleiotropic effects in treating several chronic diseases, the major limitation is its bioavailability, which affects the clinical efficacy. The low peripheral and local tissue levels of Cur may be due to poor absorption, rapid metabolism, and rapid systemic elimination.¹⁸ For this reason, using the major metabolites of Cur could

be a possible strategy to solve the problem of poor bioavailability of Cur. Tetrahydrocurcumin (THC) is one of the major metabolites of Cur and is bioavailable after administration in animals. THC was detected in various tissues and plasma for an extended period and has a longer half-life than Cur.¹⁹ Furthermore, previous studies have shown that THC had more potent antioxidant and anti-inflammatory activities than Cur.²⁰⁻²³ However, there are limited reports demonstrating the effect and function of THC on chronic airway inflammatory diseases, such as asthma. Because of the advantages of THC, the current study sought to investigate the efficacy of dietary THC on allergic asthma in an animal model and compared it to Cur.

2 | MATERIALS AND METHODS

2.1 | Animal models

Female BALB/c mice (4 weeks of age) were obtained from the Guangdong Medical Laboratory Animal Centre (Guangzhou, China) and were housed in a pathogen-free facility. The ovalbumin (OVA)-induced mouse model was established in two steps: (a) the mice were sensitized on Days 0, 7, and 14 by intraperitoneal (i.p.) injection of 40 μ g Grade V OVA (Sigma-Aldrich, St. Louis, MO, USA) and 2 mg of aluminium hydroxide (Sigma-Aldrich) in 200 μ L of pyrogen-free phosphate-buffered saline (PBS, pH = 7.3); and (b) then, from Days 21 to 25, the mice were challenged daily by aerosolized 5% OVA and subsequently administered 20 μ L OVA solution (40 mg/mL) intranasally. The mice were fed with AIN 93G diet (MediScience Ltd., Jiang Su, China) and were randomly divided into four groups (13 mice per group): AIN 93G diet with PBS only (vehicle control group), OVA-induced group, OVA plus Cur (Sigma) group (supplemented with 120 mg/kg of Cur per day, and 1 kg of the AIN 93G diet contained 800 mg of Cur, Day 0 to Day 25), and OVA plus THC (Toronto Research Chemicals, Toronto, ON, Canada) group (supplemented with 120 mg/kg of THC per day, and 1 kg of the AIN 93G diet contained 800 mg of THC, Day 0 to Day 25). Allergic symptoms were evaluated by measuring the frequency of nasal rubbing after the last OVA challenge. Bronchoalveolar lavage fluids (BALF) and mouse lung tissues were collected, and the tissues were processed into formalin-fixed paraffin-embedded blocks, tissue homogenates, tissue extracts, and single cell suspensions. The procession procedures and histo/immunohistochemistry analysis are described in the Appendix S1.

The number of mice from each group used in the experimental analysis is shown in Table E1 in Appendix S1. All experiments were approved by the Institutional Animal Care and Ethics Committee of the Sun Yat-sen University(2017-002).

2.2 | Analysis of Cur and THC levels in plasma and lung tissues

Cur and THC in plasma and lung homogenates of mice were analysed by ultra-high-performance liquid chromatography quadrupole time-of-flight mass spectrometry (UHPLC/Q-TOF-MS) as described previously with modification.²⁴ The details of sample preparation and liquid chromatography/mass spectrometry analysis are described in the Appendix S1. Linearity was acquired using calibration curves between the concentration (X) and peak area (Y). The linear regression equation was $Y = 16.496 X + 34.489$ with a correlation coefficient (r) of 0.9980. Then, the established method was applied to measure THC in plasma and lung homogenate.

2.3 | Measurement of peripheral blood cells

The numbers of red blood cells, platelets, and white blood cells (WBC) as well as the percentages of different types of WBC were analysed using an automated haematology analyser (HEMAVET 950; Drew Scientific, Waterbury, CT, USA).

2.4 | Measurements of cytokine concentrations and oxidative stress factors

Concentrations of IL-4, IL-5, IL-13, IFN- γ , IL-17A, and IL-10 were analysed using corresponding ELISA kits (eBioscience, San Diego, CA, USA) in BALF or tissue homogenates. Superoxide dismutase (SOD), glutathione (GSH), and malondialdehyde (MDA) were determined in the lung homogenates and tissue extracts using the SOD, GSH, and MDA assay kits (Beyotime, Nantong, China), respectively, according to the manufacturers' instructions. The levels of intracellular reactive oxygen species (ROS) were determined using an ROS assay kit (Beyotime) based on the DCFH-DA fluorescent probe and measured by flow cytometry.

2.5 | T cell subsets analysed by flow cytometry assay

The single-cell suspension of lungs and spleens were stimulated with Cell Stimulation Cocktail (including phorbol 12-myristate 13-acetate

(PMA), ionomycin, brefeldin A, and monesin) (eBioscience). Cells were stained with panels of monoclonal antibodies (Table E2 in Appendix S1) to determine the different T cell subsets, including Th1 (CD4⁺ IFN- γ ⁺), Th2 (CD4⁺ IL-4⁺), Th17 (CD4⁺ IL-17⁺), Tc1 (CD8⁺ IFN- γ ⁺), Tc2 (CD8⁺ IL-4⁺), Tc17 (CD8⁺ IL-17⁺), and Treg (CD4⁺ CD25⁺ Foxp3⁺) cells. All data were acquired in a FACSCalibur (BD, San Jose, CA, USA) and analysed by FlowJo software (Treestar, San Carlos, CA, USA).

2.6 | Western blot, immunohistochemistry, and quantitative PCR

Protein expressions of IL-4R α , JAK1, pJAK1, STAT6, pSTAT6, GATA3, Jagged1, Jagged2, Notch1 intracellular domain (NICD1), Notch2 intracellular domain (NICD2), Notch1, and Notch2 were determined by Western blots and immunohistochemistry. CD68 and CD11c were stained using immunohistochemistry to identify macrophages and dendritic cells, respectively. mRNA expression of cytokines (IL-4, IL-5, IL-13, IFN- γ , IL-17A, IL-33, IL-9, and IL-10) and transcription factors (GATA3, T-bet, RORc, and Foxp3) were determined by quantitative PCR. Further details can be found in the Appendix S1.

2.7 | Statistical analysis

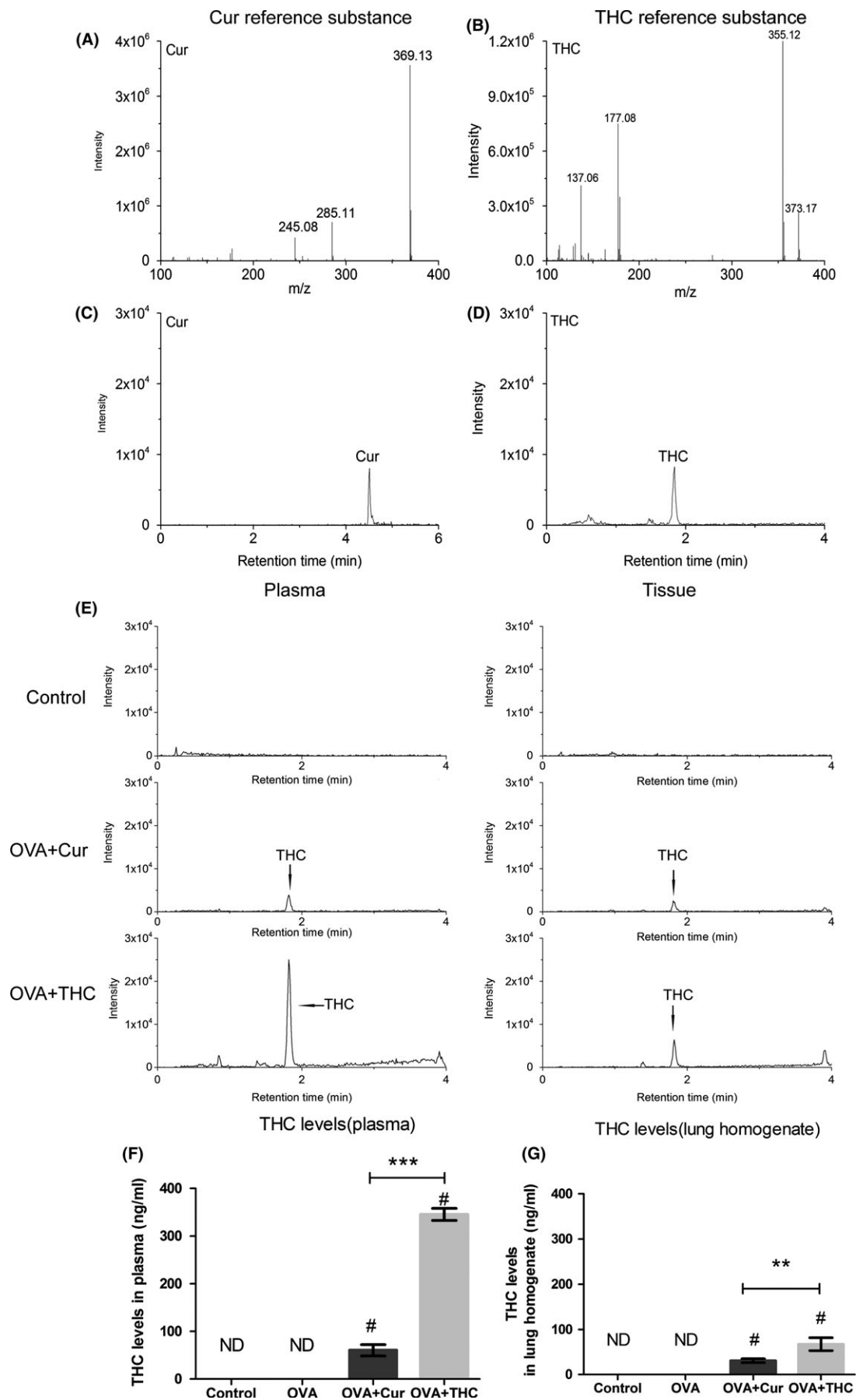
Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by the Tukey-Kramer *post hoc* test for multiple comparisons. Statistical analysis was performed using SPSS software (International Business Machines Corp. Armonk, NY), and $P < 0.05$ was considered statistically significant.

3 | RESULTS

3.1 | Enhanced bioavailability of THC in an allergic asthma mouse model treated with THC compared to Cur

The plasma and tissue levels of Cur and THC were measured in the control, OVA-induced group, and Cur- or THC-treated groups using UHPLC/Q-TOF-MS. Standard references of Cur and THC were acquired by monitoring the pairs of ion transitions of Cur (mass-to-charge ratio (m/z) 369.1 $\rightarrow$ 285.1) and THC (m/z 373.2 $\rightarrow$ 137.1) (Figure 1). Representative multiple reaction monitoring (MRM) chromatograms showed that only Cur- or THC-treated mice presented a THC signal in plasma and tissue samples, while no Cur was detected

FIGURE 1 Quantification of Cur and THC using a UHPLC/Q-TOF-MS technique. Positive ion mode was selected for compound detection, and Cur and THC produced protonated ions at m/z 369.1 and 373.2, respectively. Subsequently, the protonated ions were selected as precursor ions and fragmented to obtain the product ions at m/z 285.1 and 137.1, respectively. Then, the tandem mass spectra of Cur (A) and THC (B) references were obtained. Quantification of the Cur and THC standard references was performed by monitoring the ion transitions of Cur (m/z 369.1 $\rightarrow$ m/z 285.1) (C) and THC (m/z 373.2 $\rightarrow$ m/z 137.1) (D) by UHPLC/Q-TOF-MS chromatograms. Representative chromatogram plots of THC levels in plasma and lung homogenates of mice from the control and Cur- and THC-treated groups (E). Comparison of THC levels in plasma and lung homogenates across the four studied groups (F, G). Data are shown as the mean $\pm$ SEM ($n = 6$), # $P < 0.05$ vs control; ** $P < 0.01$ *** $P < 0.001$. The data were analysed using one-way analysis of variance (ANOVA) followed by the Tukey-Kramer *post hoc* test for multiple comparisons



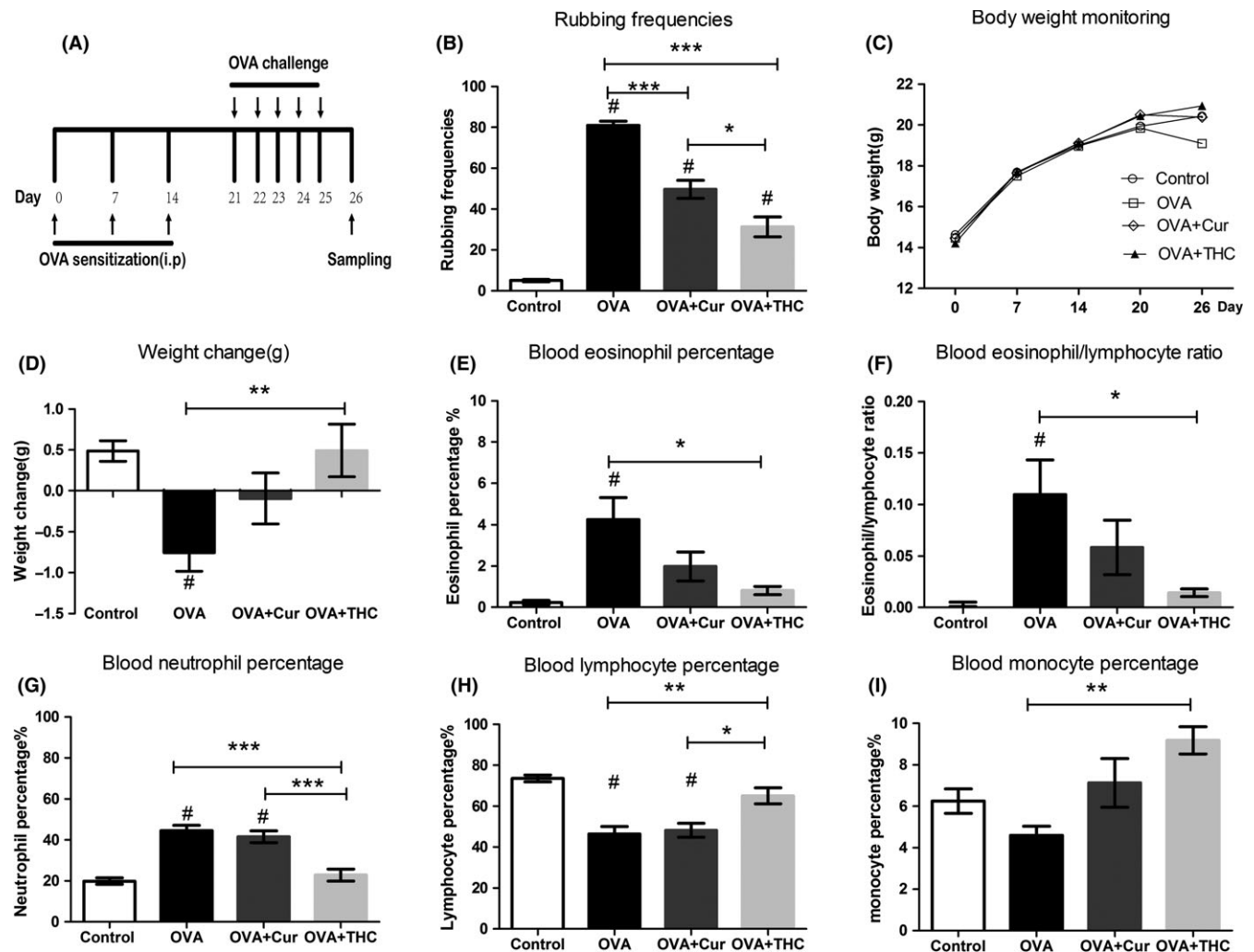


FIGURE 2 Effects of Cur and THC on nasal symptoms, body weight, and peripheral blood cells in OVA-induced mice. A, An allergic asthmatic mouse model was induced by intraperitoneal (i.p.) OVA sensitization and intranasal OVA challenges. Controls were sham-sensitized and challenged with PBS instead of OVA. B, Nasal rubbing frequencies in each group. C, The body weights of mice were recorded during the experiments on Days 0, 7, 14, 20, and 26. D, The changes of body weights between the day before and after OVA challenge. E, F, G, H, I, Blood eosinophil percentage, eosinophil/lymphocyte ratio (ELR), neutrophil percentage, lymphocyte percentage, and monocyte percentage of each group of mice. Data are shown as the mean $\pm$ SEM ($n = 8$), # $P < 0.05$ vs control; * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$. The data were analysed using one-way analysis of variance (ANOVA) followed by the Tukey-Kramer *post hoc* test for multiple comparisons

in all groups (Figure 1). Moreover, THC concentrations in plasma from the Cur- and THC-treated groups were 60.46 and 345.60 ng/mL, respectively, while THC levels in tissues from the Cur- and THC-treated groups were 30.91 and 67.28 ng/mL, respectively (Figure 1). The results demonstrated that the bioavailability of THC was higher in plasma than in tissue samples and that THC treatment could significantly enhance the THC amount in mice compared to the Cur treatment.

3.2 | THC ameliorates the changes in nasal symptoms, body weight, and circulating eosinophils in an allergic asthma mouse model

Both THC and Cur significantly relieved the nasal symptoms in asthmatic (OVA-induced) mice as indicated by observing the frequency

of nasal rubbing, and the effect of THC in reducing the rubbing frequencies was more significant than that of Cur (Figure 2). Furthermore, bristles around the nose were barely observed in the asthmatic groups, but the bristles were present in the mice treated with Cur or THC (Figure S1). The above observations indicate that THC treatment may have better effects in attenuating the symptom severity than Cur.

The body weight of the mice from the control, asthmatic, Cur-treated, and THC-treated groups was monitored throughout the experiment. Cur or THC intake did not change the body weight before OVA stimulation (by D20), while after OVA challenge, the weight of asthmatic mice was lower than that of the other groups (not statistically significant) (Figure 2). We further compared the changes in body weight on the day before and after OVA challenge and found that the weight was reduced 3.84% in asthmatic mice,

and only THC treatment could significantly reverse the reduction in body weight (6.51% recovery) (Figure 2).

One recent study showed that blood eosinophil counts and eosinophil/lymphocyte ratio (ELR) could predict local eosinophilic inflammation in patients with asthma.²⁵ Our results showed that the total white blood cell count, the percentage of blood basophils, eosinophils, and neutrophils, and the ELR were elevated in the asthmatic group compared with controls (Figure 2 & S2). More importantly, only the asthmatic mice treated with THC showed significantly reduced eosinophil or neutrophil percentages and ELR, while the effect of Cur in suppressing circulating eosinophils or neutrophils was not significant compared to that of the OVA-induced group. The percentages of blood lymphocytes and monocytes were lower in asthmatic mice, but they were up-regulated by THC treatment, while the red blood cells and platelets were not changed among the four groups (Figure S2).

3.3 | THC attenuates infiltrations of lung eosinophils, macrophages and dendritic cells, goblet cell hyper-production, and collagen deposition

The local tissue pathological changes were evaluated in the asthmatic mice and Cur- or THC-treated mice. The OVA-challenged mice showed significant tissue eosinophilia, up-regulation of macrophages (CD68⁺) and dendritic cells (CD11c⁺), increased mucus production, and a higher level of collagen deposition compared to the control group (Figure 3). Both Cur and THC treatment could relieve the eosinophil and macrophage infiltration, mucus production, and collagen formation, but the reductions in eosinophils (THC vs OVA, 86.32% decrease; Cur vs OVA, 54.69% decrease), macrophages (THC vs OVA, 64.66% decrease; Cur vs OVA, 48.07% decrease), and goblet cell production (THC vs OVA, 89.60% decrease; Cur vs OVA, 48.52% decrease) were more significant in the THC-treated group than in the Cur group (Figure 3). Regarding dendritic cells, only THC treatment had a suppressive effect in asthmatic mice (THC vs OVA, 40.38% decrease).

3.4 | THC has antioxidant effects and alleviates Th2 cytokine production in the lung tissues or BALF from asthmatic mice

The oxidative stress factors were measured in the lung tissue homogenates (extracellular space) and the cell lysates (intracellular space) from the four groups. ROS and MDA were the pro-oxidant indicators, and GSH and SOD were the antioxidant markers. MDA was not detected in the tissue homogenates, while the SOD and GSH levels were similar across the control, asthmatic, and Cur/THC-treated groups (Figure 4). Intracellular ROS and MDA production were significantly higher in the asthmatic group than the controls, and Cur or THC treatment could only reduce the MDA level; moreover, GSH, but not SOD, was suppressed in asthma, while the GSH level was recovered in the THC-treated group (Figure 4).

The production of Th2-signature cytokines (IL-4, IL-5, and IL-13) as well as the Th17-signature cytokine (IL-17) was elevated in the BALF from the asthmatic group compared to the controls (Figure 4). Both Cur and THC treatment could significantly reduce IL-13 production, while the levels of IL-4 (THC vs OVA, 70.64% decrease), IL-5 (THC vs OVA, 86.86% decrease), and IL-17 (THC vs OVA, 60.10% decrease) were only significantly suppressed in THC-treated mice. The IL-4, IL-5, IL-13, and IL-17 levels in the THC group almost reached normal levels (Figure 4). The levels of the Th1-signature cytokine IFN- γ and the cytokine (IL-10) with an immune regulation function were not different in the control, asthmatic, and Cur- and THC-treated groups.

3.5 | THC modulates different Th or Tc responses in the lung tissues from asthmatic mice

Flow cytometry analysis was performed to evaluate the amount of Th subsets (assessed by CD3⁺ CD4⁺ cells) and cytotoxic T cell (Tc) subsets (assessed by CD3⁺ CD8⁺ cells) in the lung tissues from the control, asthmatic, Cur, and THC groups. Significantly elevated percentages of Th2 (CD3⁺ CD4⁺ IL-4⁺), Th17 (CD3⁺ CD4⁺ IL-17⁺), Tc2 (CD3⁺ CD8⁺ IL-4⁺), and Tc17 (CD3⁺ CD8⁺ IL-17⁺) cells were observed in the asthmatic group compared to the control group, while the Th17, Tc2, and Tc17 percentages were significantly suppressed by treatment with Cur or THC (Figures 5 and 6). However, only THC significantly reduced the Th2 subset (58.15% decrease) in asthmatic mice (Figure 5). The Th1, Tc1 (CD8⁺ IFN- γ ⁺), or Treg (as well as IL-10⁺ Treg) percentages were similar among the four studied groups (Figures 5 and 6 and Figure S3). These Th, Tc, and Treg subsets were also analysed in the spleen tissues, and except for the increase in the Tc2 subset in the asthmatic group, there were no differences in the other subsets among the four groups (Figure S4). Taken together, the above results suggest that Cur and THC treatments may have local but not systemic effects on different Th and Tc responses, and THC more potently suppressed the Th2 subset in the lung than Cur.

3.6 | THC modulates the mRNA levels of Th2-associated genes in the lung tissues from asthmatic mice

mRNA levels of Th1-, Th2-, Th17-, and Treg-associated cytokines and transcription factors were further evaluated in the lung tissues from the OVA-induced group and THC/Cur-treated groups (Figure S5). Cytokines related to Th2 response (IL-4, IL-5, and IL-13) as well as a Th2 cell transcription factor (GATA3) were all elevated in the asthmatic mice, but only IL-13 and GATA3 were inhibited by THC treatment, and their levels in THC-treated mice were similar to those in the healthy mice. Other Th2-related cytokines (IL-33 and IL-9) and Th1-related markers (IFN- γ and T-bet) were not different in the four groups of mice. With regard to Th17 and Treg markers, only the transcription factors (RORc and Foxp3) were up-regulated in the

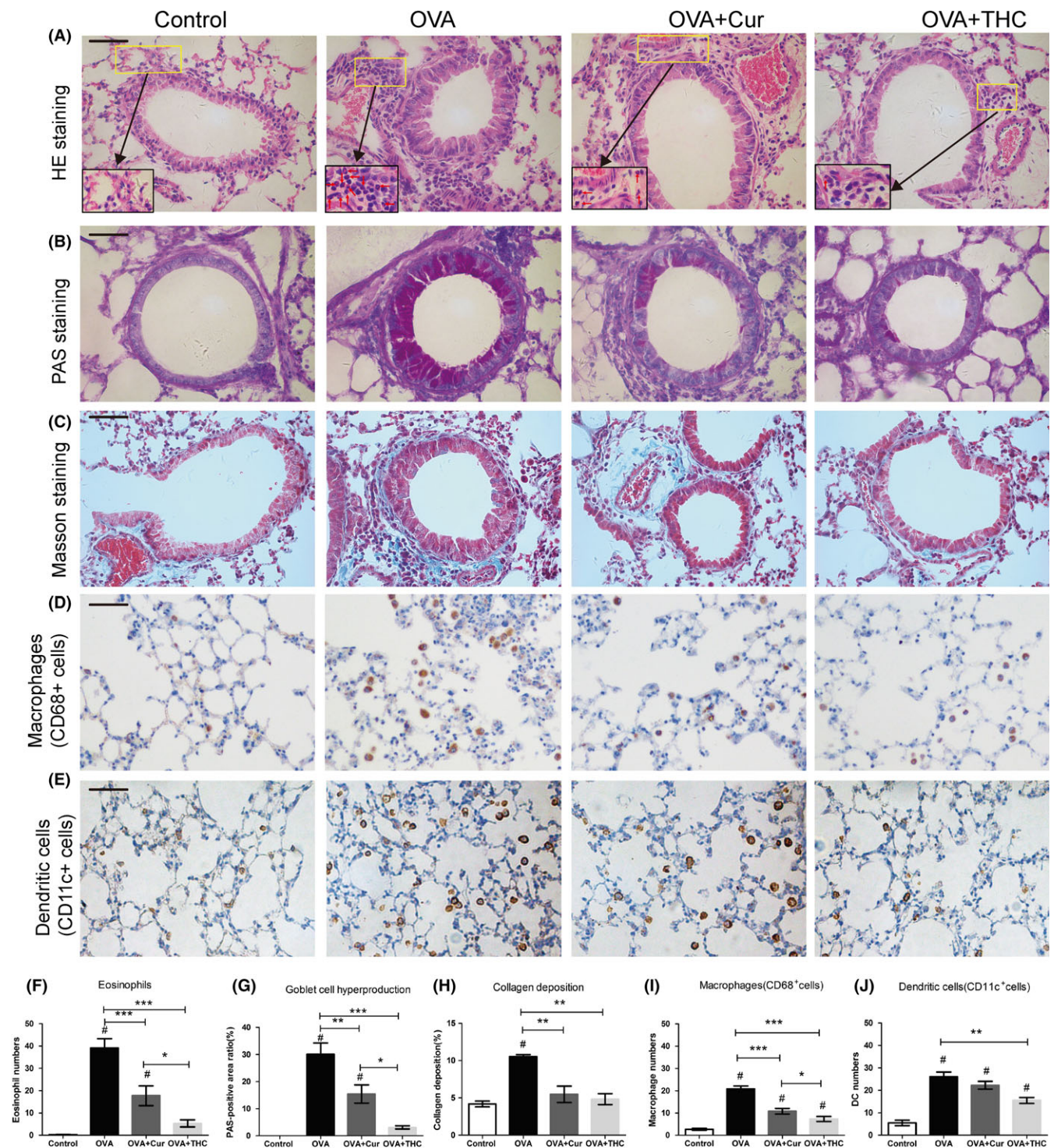


FIGURE 3 Effects of Cur and THC on histopathological changes in OVA-induced mice. Representative pictures of H&E staining (A), PAS staining (B), Masson staining (C), and immunohistochemistry staining (CD68⁺ macrophages, D; CD11c⁺ dendritic cells, E) are shown for the control, OVA-induced, OVA + Cur, and OVA + THC groups. The photos in the left bottom of panel (A) are enlargements of the indicated areas, and the red arrows point to eosinophils. Original magnification $\times 400$, scale bar = 50 μ m. The bar charts demonstrate the differences between eosinophil infiltration (F), goblet cell mucus production (G), collagen deposition (H), macrophage infiltration (I), and dendritic cell infiltration (J) among the four groups. Data are shown as the mean $\pm$ SEM ($n = 8$), # $P < 0.05$ vs control; * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$. Data were analysed using one-way analysis of variance (ANOVA) followed by the Tukey-Kramer *post hoc* test for multiple comparisons

asthmatic group compared to the control group, while the related cytokines (IL-17 and IL-10) were not different, and neither THC nor Cur treatment changed the mRNA levels of Th17 and Treg genes in

the asthmatic group. The discrepancy between the mRNA levels and protein levels (assessed by ELISA) of some Th genes (eg, IL-4, IL-5, and IL-17) was primarily attributed to the detection methods in

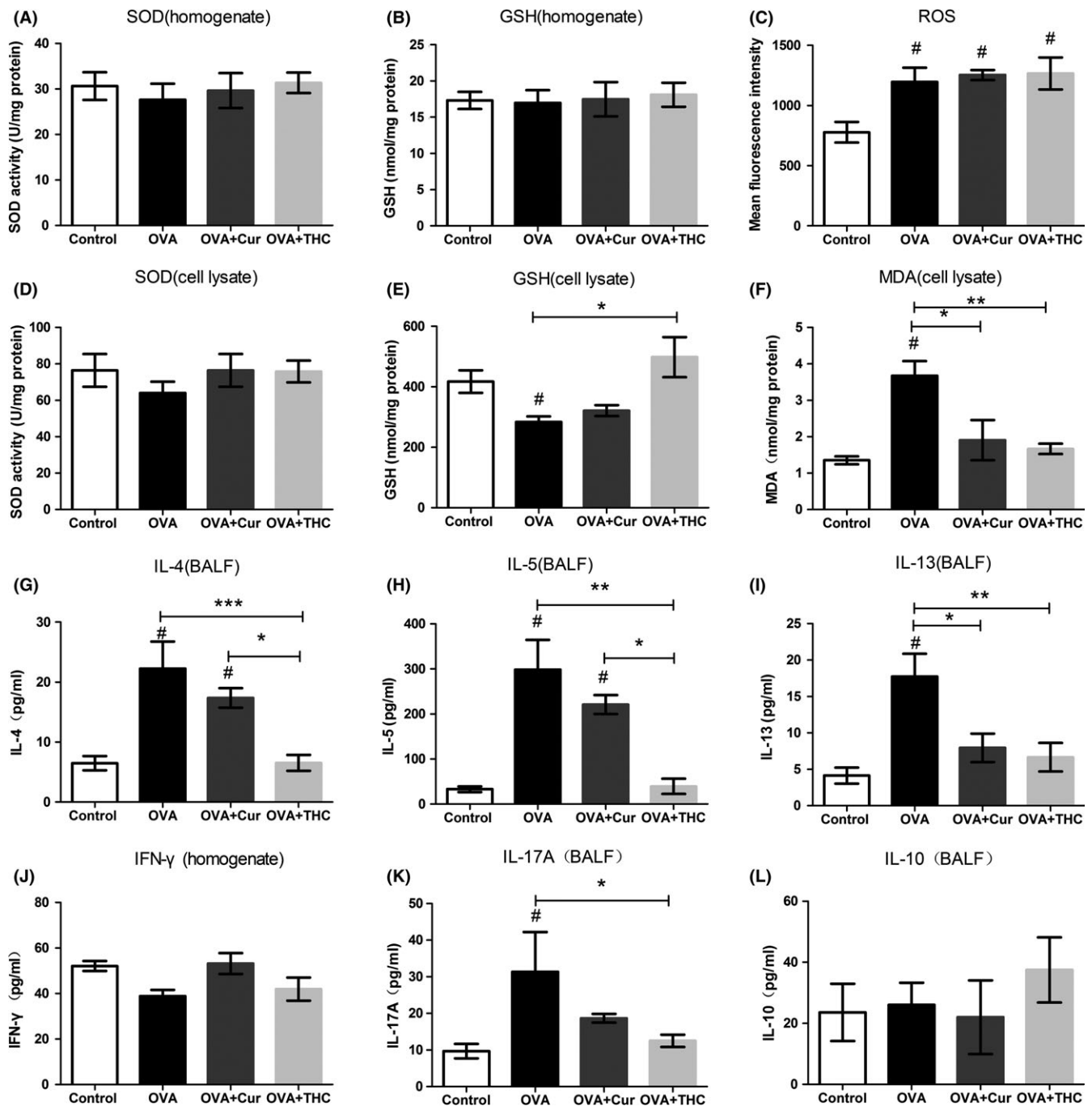


FIGURE 4 Effects of Cur and THC on oxidative stress and cytokine production in OVA-induced mice. The levels of anti-oxidant factors (GSH & SOD) (A, B, D, E) and pro-oxidant factors (MDA) (F) were determined in tissue homogenates and tissue extract. ROS were measured by flow cytometry (C). Th2 cytokines IL-4 (G), IL-5 (H), IL-13 (I), Th17 cytokine IL-17 (K), and regulatory cytokine IL-10 (L) were measured in the BALF, while Th1 cytokine IFN- γ (J) was detected in lung homogenates. Data are shown as the mean $\pm$ SEM. # P < 0.05 vs control; * P < 0.05 ** P < 0.01 *** P < 0.001. Data were analysed using one-way analysis of variance (ANOVA) followed by the Tukey-Kramer *post hoc* test for multiple comparisons

which qPCR was used to measure intracellular mRNA but ELISA was used to measure extracellular protein, and these cytokine proteins were mostly in the secreted form under the disease and treatment conditions. Nonetheless, THC, but not Cur, still had a potent inhibitory effect on mRNA expression of major Th2-related genes (IL-13 and GATA3).

3.7 | THC suppresses the IL-4R α -Jak1-STAT6 pathway in the lung tissues of asthmatic mice

The key proteins in the Th2 activation pathway IL-4R α -Jak1-STAT6^{26,27} as well as the Th2 cell transcription factor GATA3 were evaluated by western blot and immunohistochemistry assays. Protein levels of IL-

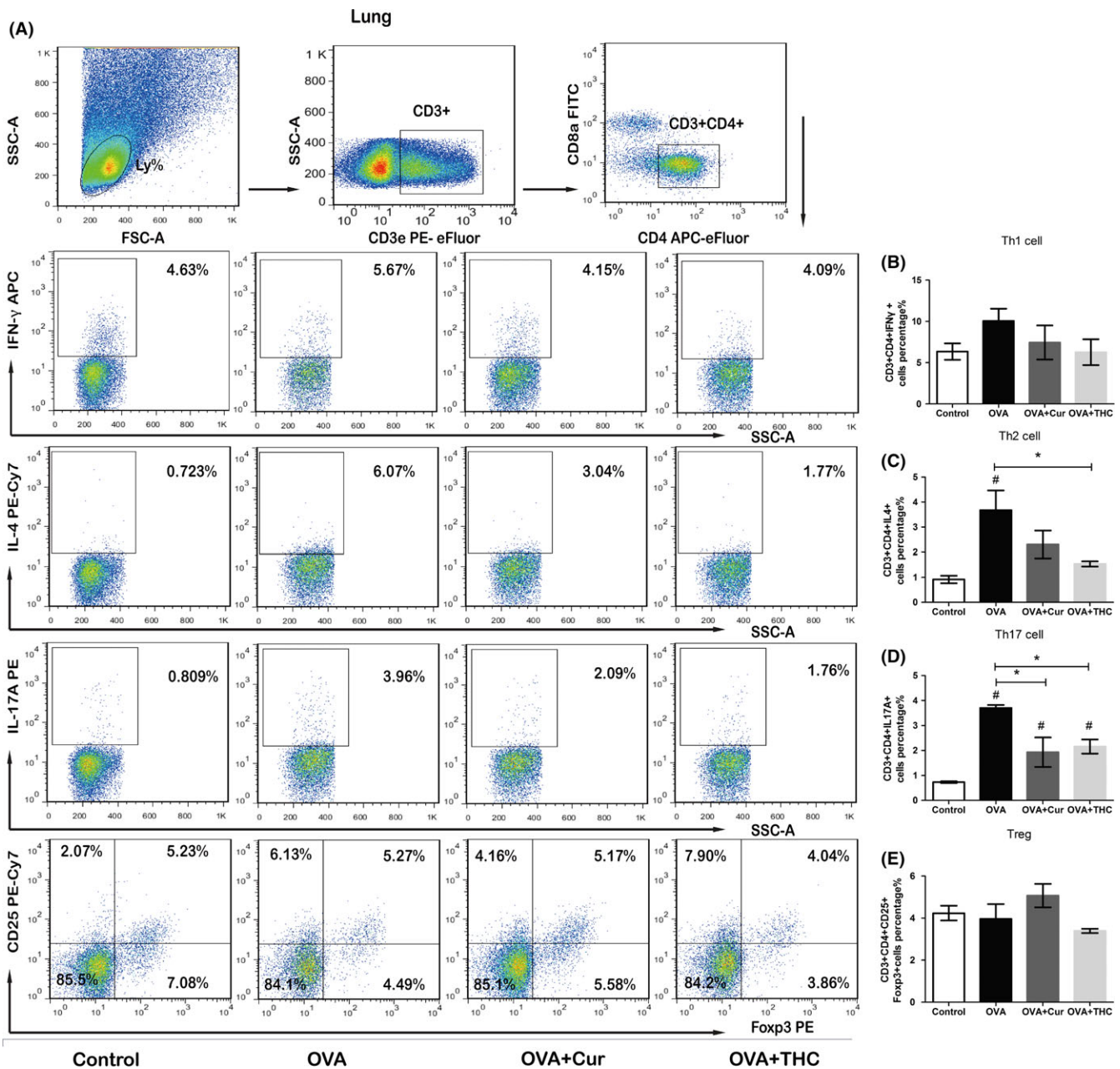


FIGURE 5 Effects of Cur and THC on modulating helper T (Th) cell subsets in the lung tissues from OVA-induced mice. Cells isolated from lung tissues were gated by a CD3⁺CD4⁺ cell population (A). Representative pictures show the changes of Th cell subsets under treatment with Cur or THC, including Th1 (CD3⁺CD4⁺IFN-γ⁺) (B), Th2 (CD3⁺CD4⁺IL-4⁺) (C), Th17 (CD3⁺CD4⁺IL-17A⁺) (D), and Treg (CD3⁺CD4⁺CD25⁺Foxp3⁺) (E). Data in the bar charts are shown as the mean ± SEM. #*P* < 0.05 vs control; **P* < 0.05 ***P* < 0.01 ****P* < 0.001. Data were analysed using one-way analysis of variance (ANOVA) followed by the Tukey-Kramer *post hoc* test for multiple comparisons

4Rα, pJAK1/Jak1, pSTAT6/STAT6, and GATA3 from the lung tissue lysates were all significantly higher in the OVA-induced group than the control group (Figure 7); treatment with THC or Cur significantly down-regulated these protein levels, while THC showed a more potent suppressive effect on IL-4Rα (THC vs OVA, 39.93% decrease; Cur vs OVA, 12.97% decrease) and GATA3 (THC vs OVA, 29.27% decrease; Cur vs OVA, 19.24% decrease) expression than Cur (Figure 7).

Immunostaining for IL-4Rα, pJAK1 and pSTAT6 was further performed in the lung tissues to localize the proteins in specific cell types. IL-4Rα, pJAK1 and pSTAT6 could be observed in epithelial cells,

endothelial cells, and leucocytes in the lung tissue sections. The percentages of IL-4Rα⁺, pJAK1⁺ or pSTAT6⁺ cells among the leucocytes (which usually infiltrated around the bronchi) were all significantly elevated in the OVA-induced group compared to the controls (Figure 7). Although both THC and Cur significantly reduced the IL-4Rα⁺, pJAK1⁺ or pSTAT6⁺ cells, THC demonstrated a stronger inhibitory effect on IL-4Rα⁺ (THC vs OVA, 84.60% decrease; Cur vs OVA, 61.74% decrease), pJAK1⁺ (THC vs OVA, 87.35% decrease; Cur vs OVA, 57.80% decrease) and pSTAT6⁺ (THC vs OVA, 73.29% decrease; Cur vs OVA, 51.06% decrease) cell infiltrations than the Cur treatment (Figure 7).

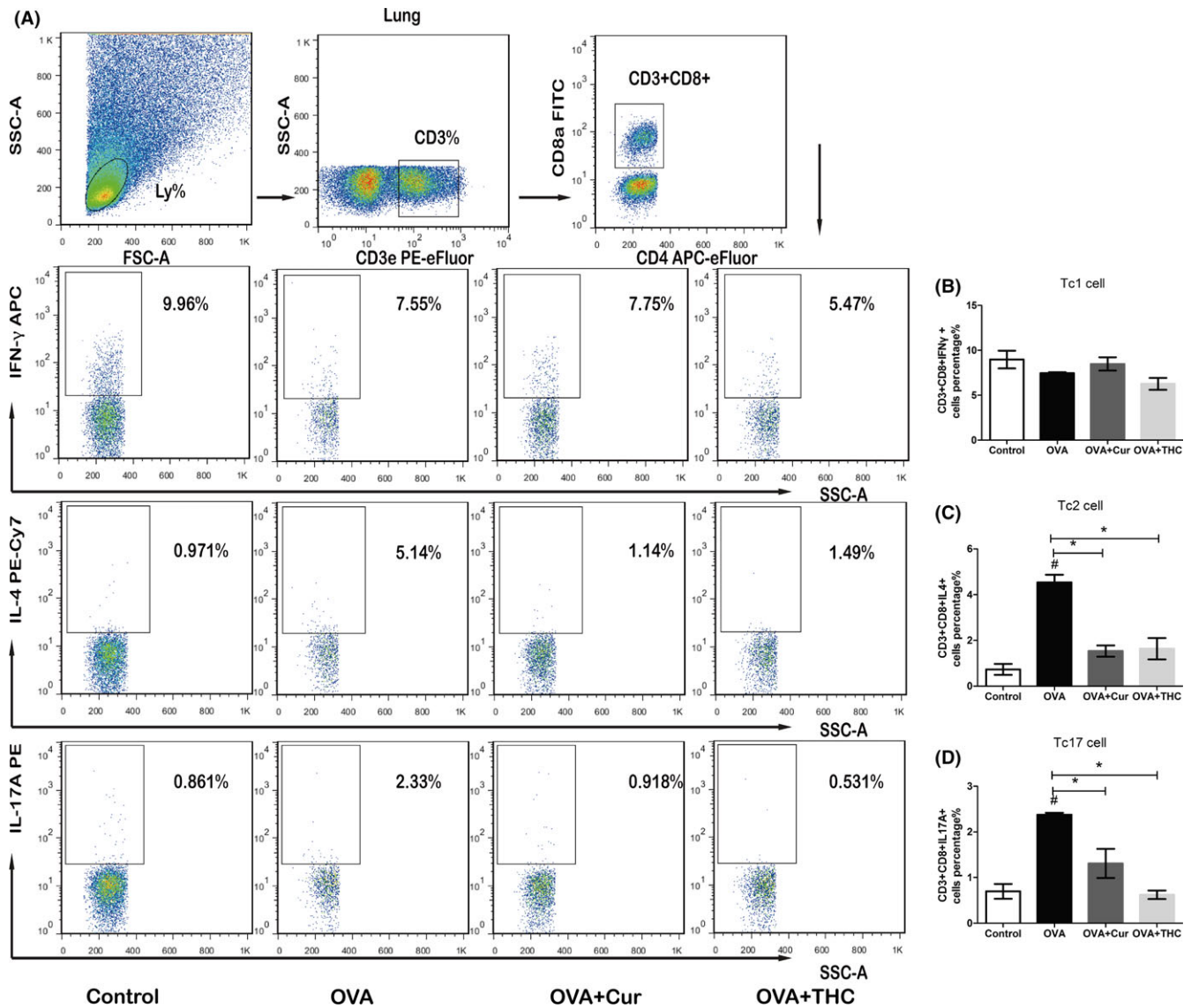


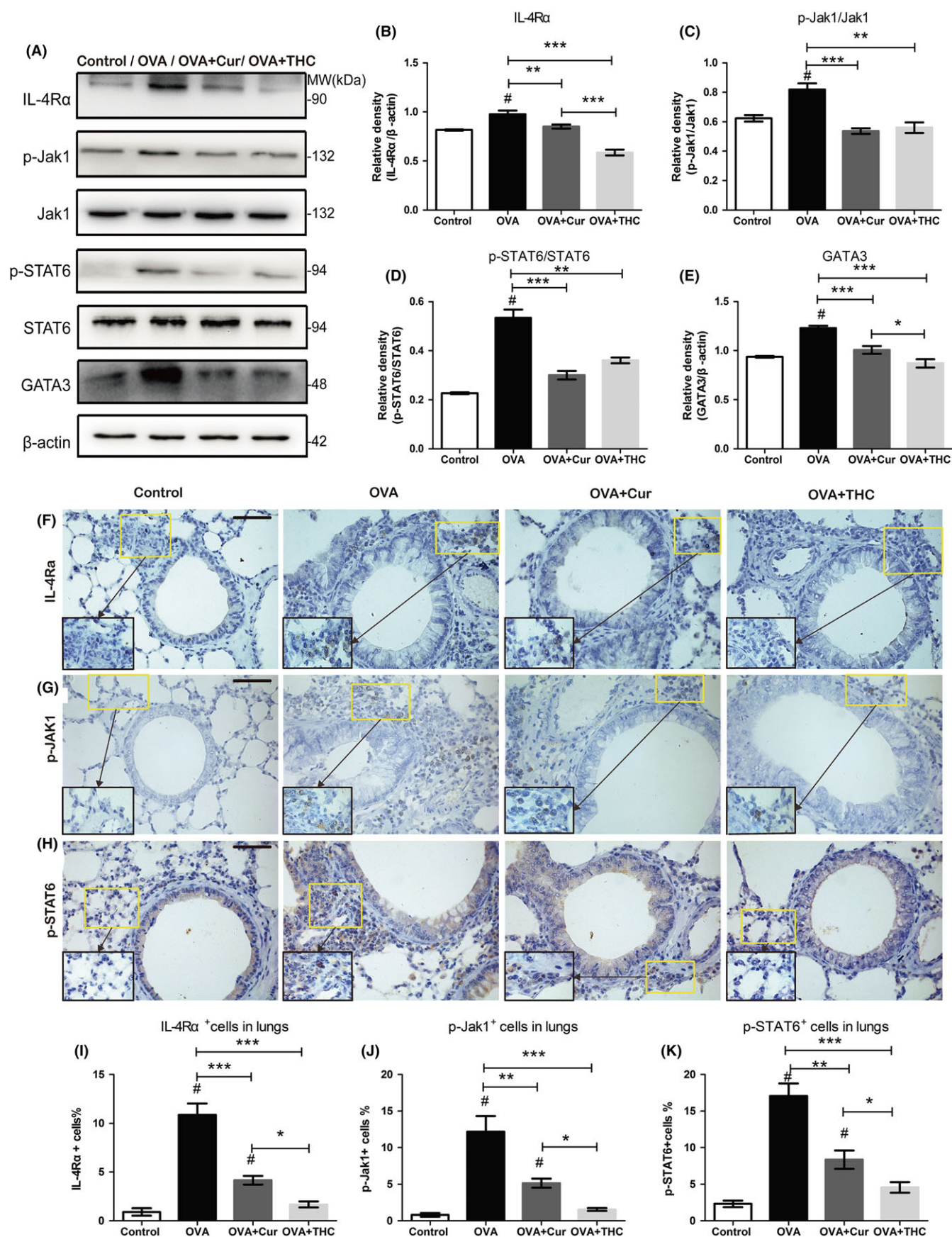
FIGURE 6 Effects of Cur and THC on modulating cytotoxic T (Tc) cell subsets in the lung tissues from OVA-induced mice. Cells isolated from lung tissues were gated by a CD3⁺CD8⁺ cell population (A). Representative pictures show the changes of Th cell subsets under treatment with Cur or THC, including Tc1 (CD3⁺CD8⁺IFN- γ ⁺) (B), Tc2 (CD3⁺CD8⁺IL-4⁺) (C), and Tc17 (CD3⁺CD8⁺IL-17A⁺) (D). Data in the bar charts were shown as the mean $\pm$ SEM. #*P* < 0.05 vs control; **P* < 0.05 ***P* < 0.01 ****P* < 0.001. Data were analysed using one-way analysis of variance (ANOVA) followed by the Tukey-Kramer *post hoc* test for multiple comparisons

3.8 | THC suppresses the Jagged1/Jagged2-Notch1/Notch2 pathway in the lung tissues of asthmatic mice

The STAT6-independent pathway Jagged1/Jagged2-Notch1/Notch2, which contributes to Th2 cell differentiation, was also analysed in the lung tissues from the different groups. Activation of Notch1/Notch2 was assessed by the Notch activated forms NICD1 (Notch intracellular domain 1) and NICD2 (Notch intracellular domain 2), respectively. The protein levels of Jagged1, Jagged 2, NICD1/Notch1, and NICD2/Notch2 were all up-regulated in tissue lysate from the OVA-induced group, while protein levels of Jagged2, NICD1/Notch1, and NICD2/Notch2 were significantly reduced in both Cur-treated and THC-treated groups, but Jagged1 was only responsive to THC

treatment (Figure 8). Furthermore, compared to Cur, THC showed a more potent suppressive effect on Jagged1 (THC vs OVA, 37.18% decrease; Cur vs OVA, 11.26% decrease), Jagged2 (THC vs OVA, 55.87% decrease; Cur vs OVA, 32.60% decrease), and NICD1/Notch1 (THC vs OVA, 45.68% decrease; Cur vs OVA, 23.46% decrease) (Figure 8).

The localization and expression patterns of Jagged1, NICD1, and NICD2 were further evaluated in the lung tissue sections. Jagged1 and NICD2 were observed in leucocytes, while NICD1 was found in both epithelial cells and leucocytes (Figure 8). The percentages of Jagged1⁺, NICD1⁺, or NICD2⁺ cells among the leucocytes as well as the density of NICD1 expression in the bronchi epithelium were all significantly enhanced in the OVA-induced group over that of the



controls (Figure 8). THC significantly reduced the Jagged1⁺ (THC vs OVA, 71.01% decrease), epithelial NICD1⁺ (THC vs OVA, 29.41% decrease), leucocyte NICD1⁺ (THC vs OVA, 62.12% decrease), and NICD2⁺ (THC vs OVA, 70.32% decrease) cells, but Cur only inhibited epithelial NICD1⁺ (Cur vs OVA, 22.54% decrease) and NICD2⁺ (Cur vs OVA, 51.61% decrease) cells (Figure 8).

3.9 | Summary and comparison of the Cur and THC effects on an allergic asthmatic mouse model

Table E3 in Appendix S1 summarizes the parameters analysed in the current results, showing significant differences between the Cur-treated and THC-treated groups and the asthmatic group and comparing the efficacy between Cur and THC treatment in regulating allergic asthma inflammation. Overall, THC significantly modulated these parameters in asthmatic mice, and furthermore, THC was more effective than Cur in relieving the nasal symptoms, recovering the body weight, suppressing the eosinophils, macrophages and mucus production, enhancing the antioxidant enzymes, inhibiting the Th2 and Th17 cytokine levels (IL-4, IL-5, IL-17) and Th2 cells, and reducing the IL-4R α -Jak1-STAT6 and Jagged1/Jagged2-Notch1/Notch2 axis activation.

4 | DISCUSSION

Allergic asthma is a chronic inflammatory disease broadly defined by bronchoconstriction, airway hyper-responsiveness, and increased inflammatory infiltrates and mucus production. Tissue eosinophilic infiltration with a prominent Th2 response is the major inflammatory phenotype in allergic asthma. Bronchodilators and inhaled/oral corticosteroids are commonly used as the first-line treatment, but their use is limited due to long-term side effects. There have been major advances in treating asthma (such as anti-IL-5/IL-13 therapy),²⁸⁻³⁰ but there are some limitations for antibody-based treatments: (a) the identification of potentially responsive patients based on these biomarkers is required, (b) adverse effects may exist after discontinuation, and (c) the cost of monoclonal antibody medication is high. Thus, there is an urgent need to develop new drugs that might be beneficial in asthma treatment strategies.

Herbal medicine has a beneficial effect on most chronic inflammatory diseases due to its pleiotropic effects in regulating the human immune system. Cur can modulate airway inflammatory disorders (such as asthma, chronic obstructive pulmonary disease, and pulmonary fibrosis) by regulating multiple targets, which are linked with its anti-inflammatory, antioxidant, and antibacterial properties.¹⁰

Although Cur has the advantages of safety, efficacy and affordability, its therapeutic effect and clinical application are hindered by its low bioavailability. Therefore, various approaches have been undertaken to improve its bioavailability, which include the use of adjuvant, liposomal Cur, Cur nanoparticles, and structural analogues.³¹ THC, which is one of the major active metabolites of Cur, was detected in tissue and plasma following oral delivery. However, whether THC is superior to Cur at the molecular level remains unclear, and previous studies have suggested that THC and Cur may have different effects on various types of diseases.³² The present study demonstrates for the first time the efficacy of THC in treating allergic asthma mice and compared its effects to those of Cur.

The current study first confirmed that only THC, not Cur, was detectable in plasma and tissues from both Cur- and THC-treated mice and that the bioavailability of THC was significantly higher in mice treated with THC than in those treated with Cur. The current results showed that THC was more effective in relieving the nasal symptoms, eosinophil infiltration, and mucus production than Cur, and only THC treatment could normalize the body weight and suppress peripheral eosinophil levels; moreover, THC, but not Cur, could down-regulate the production of the Th2 signature cytokines IL-4 and IL-5 and reduce the Th2 cell population. The inhibitory effects of THC on IL-5 levels and Th2 subsets may contribute to its more potent efficacy on eosinophil infiltration compared with that of Cur. The antioxidant effect of THC may be mediated by altering the GSH and MDA levels, while Cur could only reduce the MDA level, indicating THC exhibits higher antioxidant activity. These findings are consistent with those of previous reports.³² Collectively, these findings indicate that THC is superior to Cur in both antioxidant and anti-inflammatory properties in treating allergic asthma.

The STAT6-dependent (IL-4-IL-4R α -Jak1-STAT6) and STAT6-independent (Jagged1/Jagged2-Notch1/Notch2) pathways play crucial roles in allergic airway inflammation, including the promotion of Th2 cell polarization and mucus hyper-production.^{33,34} The current results demonstrated that the expression of IL-4R α and GATA3 and the activation (phosphorylation) of Jak1 and STAT6 were decreased in local leucocytes by THC treatment, and the inhibitory effect of THC was stronger than that of Cur with regard to the STAT6-independent pathway, Jagged1, Jagged2, and the activated forms of Notch 1 (NICD1) and Notch2 (NICD2), which were all suppressed by THC treatment. The reduction of Th2 cells in THC-treated mice shown in our results might have been partially caused by inhibition of GATA3 through the STAT6-dependent and STAT6-independent signalling pathways.

FIGURE 7 Effects of Cur and THC on regulating the IL-4R α -Jak1-STAT6 pathway and GATA3 protein expression in the lung tissues from OVA-induced mice via western blot and immunohistochemistry analysis. Representative western blot pictures of IL-4R α , p-Jak1, p-STAT6, GATA3 and total Jak1, STAT6 in lung tissue lysates (A). Protein expressions of IL-4R α (B), p-Jak1/Jak1 (C), p-STA6/STAT6 (D) and GATA3 (E) were quantified based on the density of the bands. Representative images of immunohistochemical staining for IL-4R α (F), p-Jak1 (G) and p-STAT6 (H) in lung sections. The percentages of IL-4R α ⁺ (I), pJAK1⁺ (J), and pSTAT6⁺ (K) cells of total infiltrating cells around bronchi in lung sections were determined. Data are shown as the mean $\pm$ SEM, #*P* < 0.05 vs control; **P* < 0.05 ***P* < 0.01 ****P* < 0.001. Data were analysed using one-way analysis of variance (ANOVA) followed by the Tukey-Kramer *post hoc* test for multiple comparisons

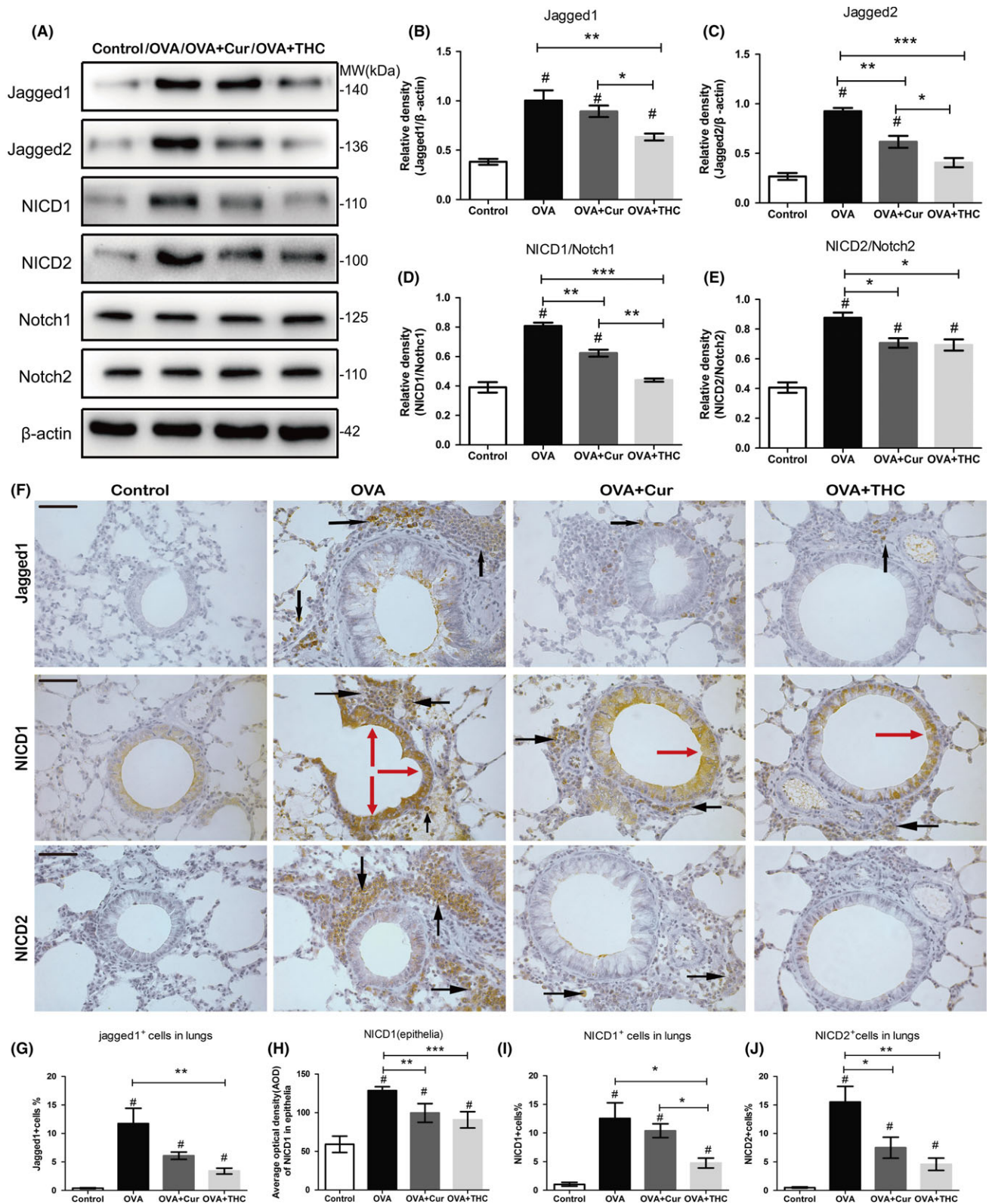


FIGURE 8 Effects of Cur and THC on regulating Jagged1/Jagged2-Notch1/Notch2 pathway protein expression in the lung tissues from OVA-induced mice via Western blot and immunohistochemistry analysis. Representative Western blot pictures of Jagged1, Jagged2, NICD1, NICD2, Notch1, and Notch2 in lung tissue lysates (A). Protein expressions of Jagged1 (B), Jagged2 (C), NICD1/Notch1 (D), and NICD2/Notch2 (E) were quantified based on the density of the bands. Representative images of immunohistochemical staining for Jagged1, NICD1 and NICD2 in lung sections (F). The percentages of Jagged1⁺ (G), NICD1⁺ (I), and NICD2⁺ (J) cells of total infiltrating cells around bronchi, and epithelial NICD1 density among the epithelial region (H) in lung sections were determined. Data are shown as the mean $\pm$ SEM, #*P* < 0.05 vs control; **P* < 0.05 ***P* < 0.01 ****P* < 0.001. Data were analysed using one-way analysis of variance (ANOVA) followed by the Tukey-Kramer post hoc test for multiple comparisons

Although THC was more effective than Cur in the asthma mouse model, Cur could reduce the inflammatory infiltrates in the lung tissue, alter the percentage of some Th subsets, and suppress IL-4R α axis activity. Our observations suggest that Cur still has beneficial effects on allergic asthmatic mice, which is concordant with the results from previous studies.³⁵ Interestingly, we found that the Th17 and Tc17 subsets were both suppressed by THC or Cur treatment, while THC or Cur did not modulate the Th1 or Treg responses. Th17 cells have high lineage plasticity and flexibility under inflammatory conditions, ie, they can further differentiate and switch to other T cell phenotypes by expressing effector cytokines (such as intermediate Th1/Th17 cells, Th1 cells and Tregs).^{36–38} These findings indicate that the THC or Cur suppression of eosinophil infiltration could be associated with the inhibition of Th2 or Th17 responses rather than regulation of the Th1 response or Th17 to Th1 or Treg cell polarization.

Collectively, our findings indicate that THC plays a crucial role in the Cur-induced biological effects and that THC is more effective than Cur. We believe that the superior effects on allergic asthma shown by THC are due to its increased bioavailability in plasma and tissues compared to that of Cur. Given that Cur is widely used as a dietary supplement and well-tolerated in humans and that THC is the major metabolite of Cur, THC should have a comparable safety profile to Cur but a better efficacy. Therefore, the current results suggest that THC could be developed as a promising nutraceutical agent for treating allergic airway inflammatory diseases, and more in-depth animal studies are required to evaluate the pharmacokinetics and long-term safety as well as to clarify the molecular mechanism of THC in modulating allergic airway inflammation. Clinical trials could also be conducted to evaluate the THC efficacy on asthma or allergic rhinitis.

In conclusion, this is the first study investigating the therapeutic effects of THC on allergic asthma in a mouse model, and the results showed that oral administration of THC significantly relieved airway inflammation by reducing the symptoms, oxidative stress, eosinophil infiltration, Th2 cytokine production, and Th2 and Th17 responses, as well as the Th2 signalling pathways (IL-4-IL-4R α -Jak1-STAT6 and Jagged1/Jagged2-Notch 1/Notch2 axes). More importantly, the anti-inflammatory efficacy of THC was superior to that of Cur on allergic inflammation, indicating THC could be an essential bioactive product to overcome the limitations of Cur. Therefore, THC's therapeutic benefits in treating allergic airway diseases are promising, and additional studies should be performed to evaluate its clinical effectiveness in humans.

ACKNOWLEDGEMENTS

This study was supported by grants from the Natural Science Foundation of Guangdong Province, China (No. 2015A050502020, No. 2017A030313621), the Guangdong Provincial Medical Science and Technology Research Fund (No. B2016005), the National Natural Science Foundation of China (No. 81500772 and No. 81700892, No. 81770983), the Fundamental Research Funds for the Central

Universities (No. 17ykzd19), and the Guangzhou Science and Technology Programme (No. 201605030003).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ORCID

Wen long Liu  <http://orcid.org/0000-0003-1296-5144>

Chun Wei Li  <http://orcid.org/0000-0001-6628-7698>

REFERENCES

1. Julia V, Macia L, Dombrowicz D. The impact of diet on asthma and allergic diseases. *Nat Rev Immunol*. 2015;15(5):308–322.
2. Nagel G, Weinmayr G, Kleiner A, Garcia-Marcos L, Strachan DP. Effect of diet on asthma and allergic sensitisation in the International Study on Allergies and Asthma in Childhood (ISAAC) Phase Two. *Thorax*. 2010;65(6):516–522.
3. Yang K, Shrestha S, Zeng H, et al. T cell exit from quiescence and differentiation into Th2 cells depend on raptor-mTORC1-mediated metabolic reprogramming. *Immunity*. 2013;39(6):1043–1056.
4. de Matos OG, Amaral SS, Marques Pereira Da Silva PE, et al. Dietary supplementation with omega-3-PUFA-rich fish oil reduces signs of food allergy in ovalbumin-sensitized mice. *Clin Dev Immunol*. 2012;2012:236564.
5. Weise C, Hilt K, Milovanovic M, Ernst D, Ruehl R, Worm M. Inhibition of IgE production by docosahexaenoic acid is mediated by direct interference with STAT6 and NF kappa B pathway in human B cells. *J Nutr Biochem*. 2011;22(3):269–275.
6. Trompette A, Gollwitzer ES, Yadava K, et al. Gut microbiota metabolism of dietary fiber influences allergic airway disease and hematopoiesis. *Nat Med*. 2014;20(2):159–166.
7. Smith PM, Howitt MR, Panikov N, et al. The microbial metabolites, short-chain fatty acids, regulate colonic T-reg cell homeostasis. *Science*. 2013;341(6145):569–573.
8. Yokota-Nakatsuma A, Takeuchi H, Ohoka Y, et al. Retinoic acid prevents mesenteric lymph node dendritic cells from inducing IL-13-producing inflammatory Th2 cells. *Mucosal Immunol*. 2014;7(4):786–801.
9. Vasilidou JE, Lui S, Walker SA, et al. Vitamin D deficiency induces Th2 skewing and eosinophilia in neonatal allergic airways disease. *Allergy*. 2014;69(10):1380–1389.
10. Kunnumakkara AB, Bordoloi D, Padmavathi G, et al. Curcumin, the golden nutraceutical: multitargeting for multiple chronic diseases. *Br J Pharmacol*. 2017;174(11):1325–1348.
11. Abidi A, Gupta S, Agarwal M, Bhalla HL, Saluja M. Evaluation of efficacy of curcumin as an add-on therapy in patients of bronchial asthma. *J Clin Diagn Res*. 2014;8(8):HC19–24.
12. Lee JH, Kim JW, Ko NY, et al. Curcumin, a constituent of curry, suppresses IgE-mediated allergic response and mast cell activation at the level of Syk. *J Allergy Clin Immunol*. 2008;121(5):1225–1231.
13. Ma C, Ma Z, Fu Q, Ma S. Curcumin attenuates allergic airway inflammation by regulation of CD4(+)CD25(+) regulatory T cells (Tregs)/Th17 balance in ovalbumin-sensitized mice. *Fitoterapia*. 2013;87:57–64.
14. Kobayashi T, Hashimoto S, Horie T. Curcumin inhibition of Der-matophagoides farinea-induced interleukin-5 (IL-5) and granulocyte macrophage-colony stimulating factor (GM-CSF) production by lymphocytes from bronchial asthmatics. *Biochem Pharmacol*. 1997;54(7):819–824.

15. Moon D, Kim M, Lee H, et al. Curcumin attenuates ovalbumin-induced airway inflammation by regulating nitric oxide. *Biochem Biophys Res Commun*. 2008;375(2):275-279.
16. Barnes PJ. Nitric oxide and airway disease. *Ann Med*. 1995;27(3):389-393.
17. Chong L, Zhang W, Nie Y, et al. Protective effect of curcumin on acute airway inflammation of allergic asthma in mice through Notch1-GATA3 signaling pathway. *Inflammation*. 2014;37(5):1476-1485.
18. Pan MH, Huang TM, Lin JK. Biotransformation of curcumin through reduction and glucuronidation in mice. *Drug Metab Dispos*. 1999;27(4):486-494.
19. Vijaya Saradhi UVR, Ling Y, Wang J, et al. A liquid chromatography-tandem mass spectrometric method for quantification of curcuminoids in cell medium and mouse plasma. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2010;878(30):3045-3051.
20. Somparn P, Phisalaphong C, Nakornchai S, Unchern S, Morales NP. Comparative antioxidant activities of curcumin and its demethoxy and hydrogenated derivatives. *Biol Pharm Bull*. 2007;30(1):74-78.
21. Naito M, Wu X, Nomura H, et al. The protective effects of tetrahydrocurcumin on oxidative stress in cholesterol-fed rabbits. *J Atheroscler Thromb*. 2002;9(5):243-250.
22. Sugiyama Y, Kawakishi S, Osawa T. Involvement of the beta-diketone moiety in the antioxidative mechanism of tetrahydrocurcumin. *Biochem Pharmacol*. 1996;52(4):519-525.
23. Okada K, Wangpoengtrakul C, Tanaka T, Toyokuni S, Uchida K, Osawa T. Curcumin and especially tetrahydrocurcumin ameliorate oxidative stress-induced renal injury in mice. *J Nutr*. 2001;131(8):2090-2095.
24. Novaes JT, Lillico R, Sayre CL, et al. Disposition, metabolism and histone deacetylase and acetyltransferase inhibition activity of tetrahydrocurcumin and other curcuminoids. *Pharmaceutics*. 2017;9(UNSP 454):45.
25. Zhang XY, Simpson JL, Powell H, et al. Full blood count parameters for the detection of asthma inflammatory phenotypes. *Clin Exp Allergy*. 2014;44(9):1137-1145.
26. Chapoval S, Dasgupta P, Dorsey NJ, Keegan AD. Regulation of the T helper cell type 2 (Th2)/T regulatory cell (Treg) balance by IL-4 and STAT6. *J Leukoc Biol*. 2010;87(6):1011-1018.
27. Chen XH, Patel BKR, Wang L, et al. Jak1 expression is required for mediating interleukin-4-induced tyrosine phosphorylation of insulin receptor substrate and Stat6 signaling molecules. *J Biol Chem*. 1997;272(10):6556-6560.
28. Murphy K, Jacobs J, Bjermer L, et al. Long-term safety and efficacy of reslizumab in patients with eosinophilic asthma. *J Allergy Clin Immunol Pract*. 2017;5(6):1572-1581.e3.
29. Choy DF, Jia G, Abbas AR, et al. Peripheral blood gene expression predicts clinical benefit from anti-IL-13 in asthma. *J Allergy Clin Immunol*. 2016;138(4):1230-1233.e8.
30. Brightling CE, Chaney P, Leigh R, et al. Efficacy and safety of tralokinumab in patients with severe uncontrolled asthma: a randomised, double-blind, placebo-controlled, phase 2b trial. *Lancet Respir Med*. 2015;3(9):692-701.
31. Anand P, Kunnumakkara AB, Newman RA, Aggarwal BB. Bioavailability of curcumin: problems and promises. *Mol Pharm*. 2007;4(6):807-818.
32. Aggarwal BB, Deb L, Prasad S. Curcumin differs from tetrahydrocurcumin for molecular targets, signaling pathways and cellular responses. *Molecules*. 2015;20(1):185-205.
33. Chatila TA. Interleukin-4 receptor signaling pathways in asthma pathogenesis. *Trends Mol Med*. 2004;10(10):493-499.
34. Amsen D, Antov A, Flavell RA. The different faces of Notch in T-helper-cell differentiation. *Nat Rev Immunol*. 2009;9(2):116-124.
35. Lelli D, Sahebkar A, Johnston TP, Pedone C. Curcumin use in pulmonary diseases: state of the art and future perspectives. *Pharmacol Res*. 2017;115:133-148.
36. Yang B, Floess S, Hagemann S, et al. Development of a unique epigenetic signature during *in vivo* Th17 differentiation. *Nucleic Acids Res*. 2015;43(3):1537-1548.
37. Mazzoni A, Santarlasci V, Maggi L, et al. Demethylation of the RORC2 and IL17A in human CD4(+) T lymphocytes defines Th17 origin of nonclassic Th1 cells. *J Immunol*. 2015;194(7):3116-3126.
38. Xu T, Stewart KM, Wang X, et al. Metabolic control of TH17 and induced Treg cell balance by an epigenetic mechanism. *Nature*. 2017;548(7666):228-233.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Chen BL, Chen YQ, Ma BH, et al. Tetrahydrocurcumin, a major metabolite of curcumin, ameliorates allergic airway inflammation by attenuating Th2 responses and suppressing the IL4Ra-Jak1-STAT6 and Jagged1/Jagged2 -Notch1/Notch2 pathways in asthmatic mice. *Clin Exp Allergy*. 2018;48:1494-1508.
<https://doi.org/10.1111/cea.13258>

Figure S2

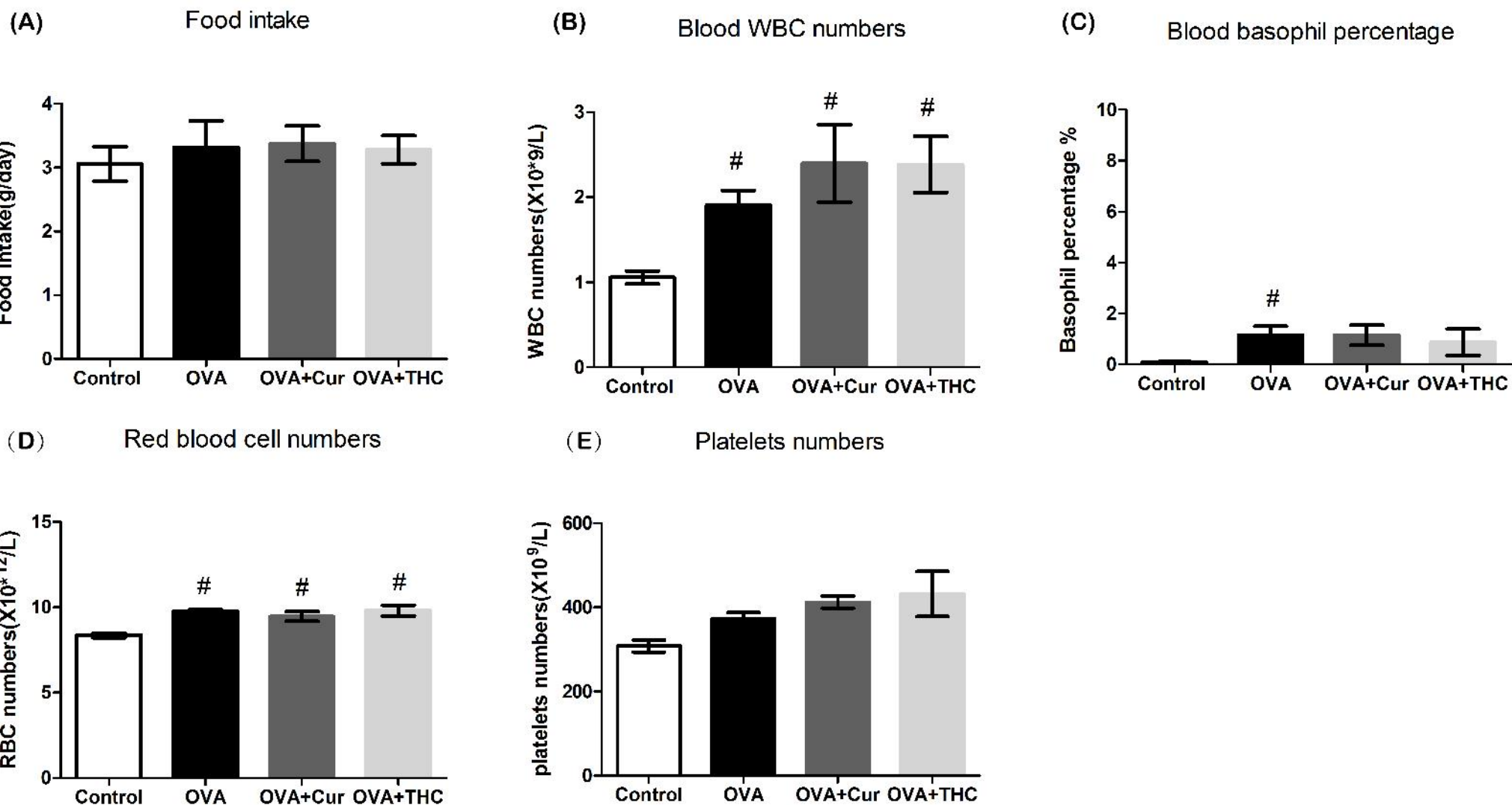
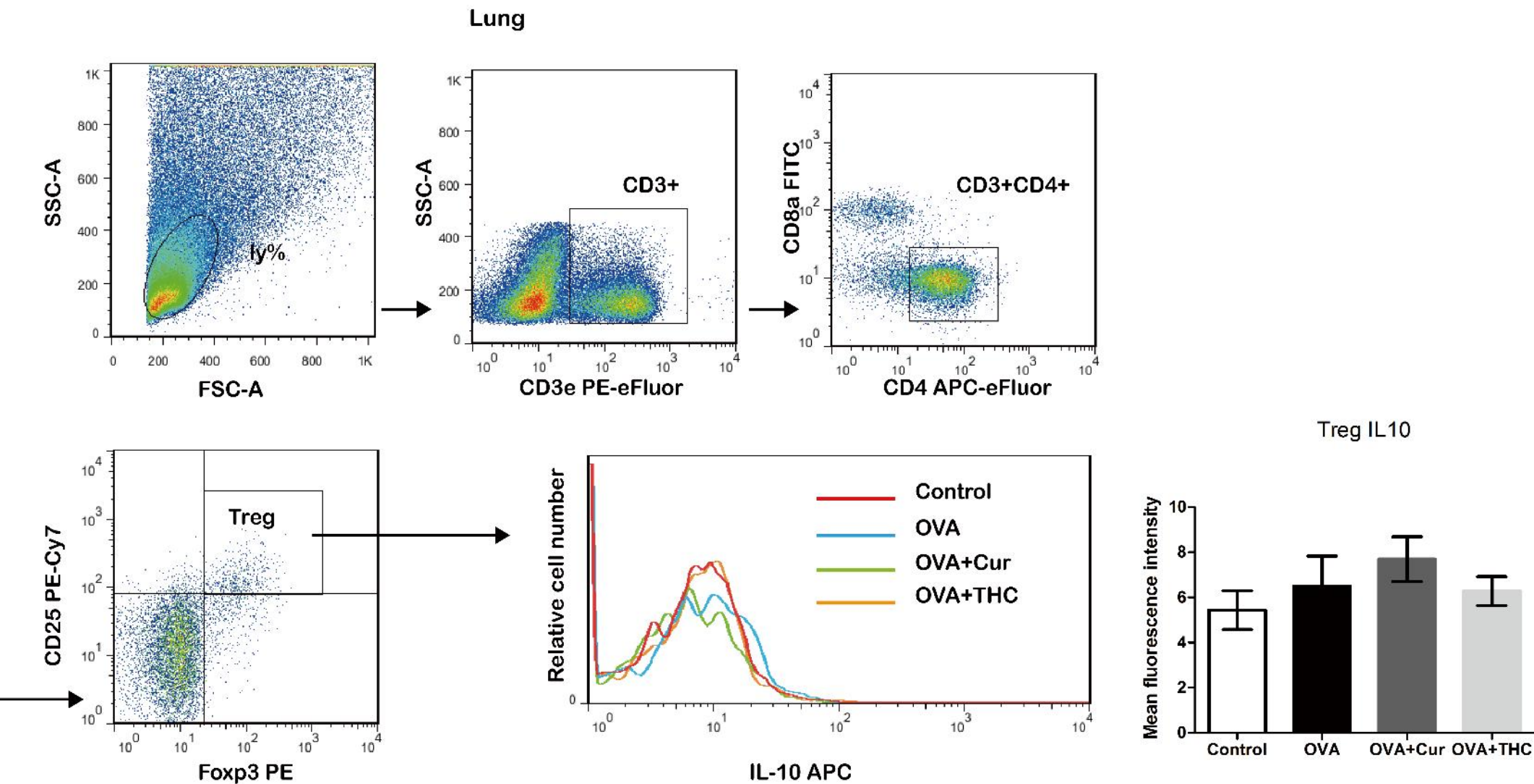


Figure S3



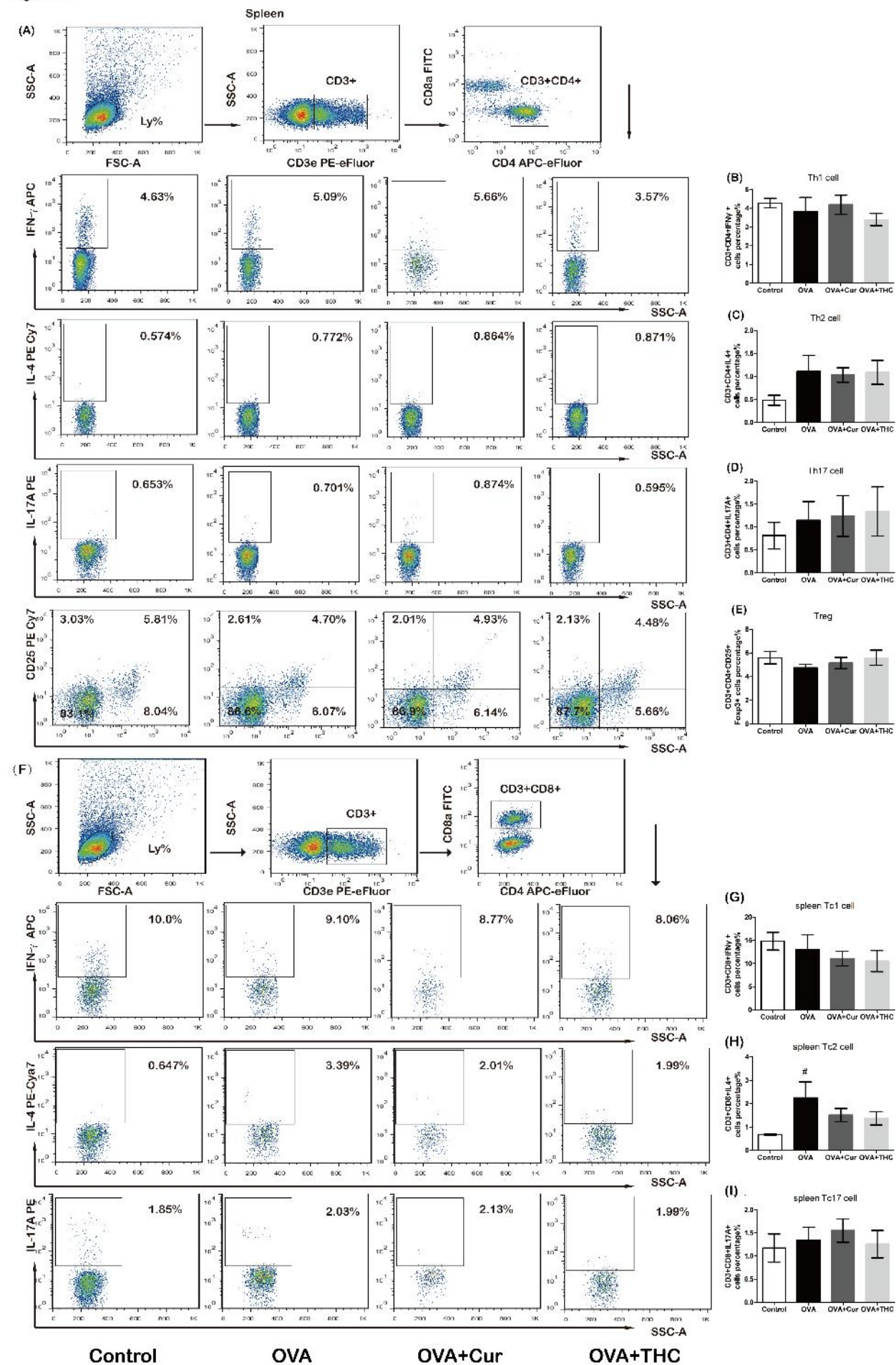
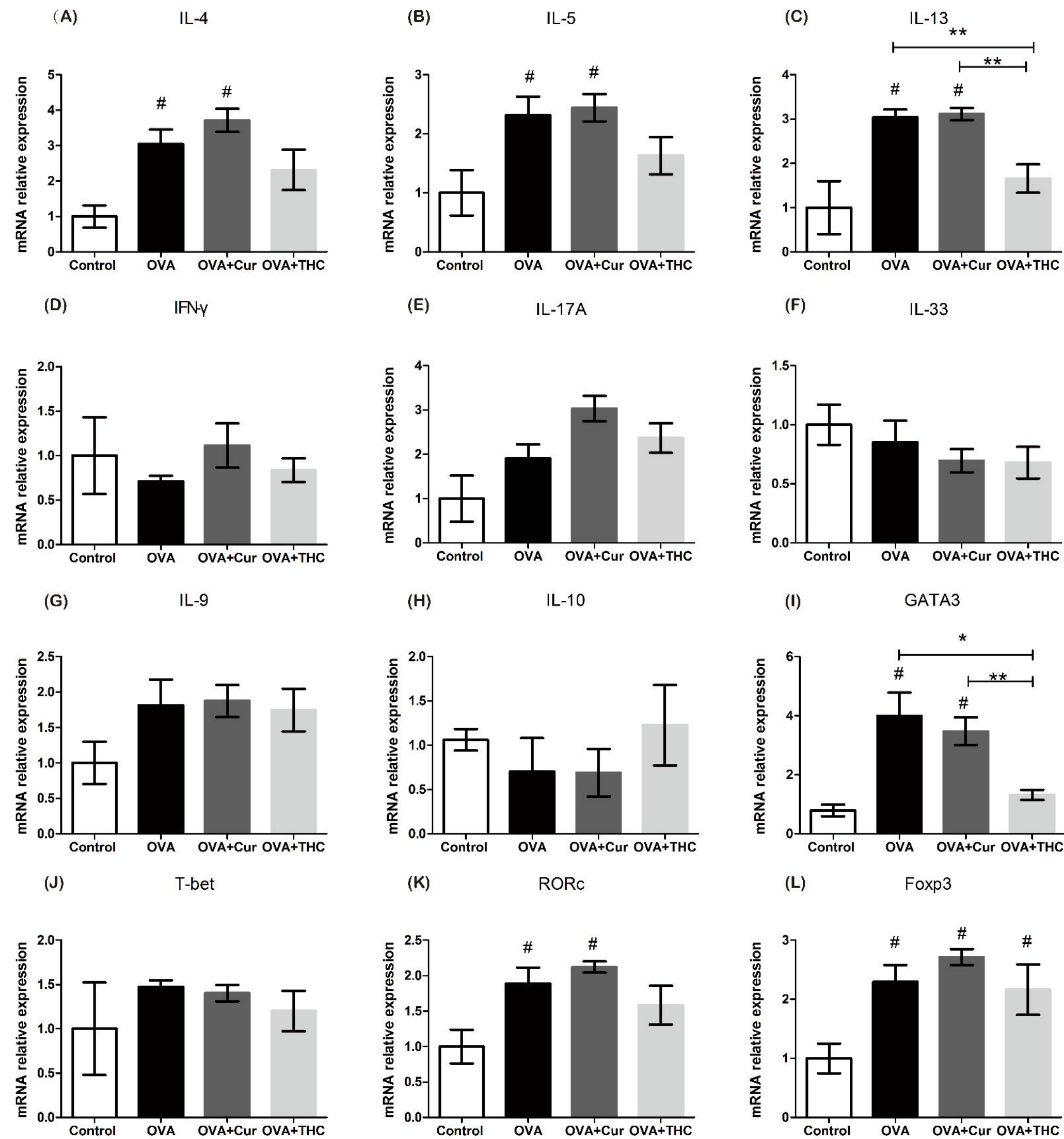


Figure S5



Supplement

Tetrahydrocurcumin, a major metabolite of curcumin, ameliorates allergic airway inflammation by attenuating Th2 responses and suppressing the IL4Ra-Jak1-STAT6 and Jagged1/Jagged2-Notch1/Notch2 pathway in asthmatic mice

Materials and Methods

Preparations of bronchoalveolar lavage fluids (BALF), lung tissue homogenates, lung tissue extract, and cell suspension from tissues

BALF was collected by gentle aspiration of PBS injected into the airways of mice via a tracheal cannula. The lungs were snap-frozen in liquid nitrogen and were homogenized with 4 times of PBS by using a Bullet Blender Blue (Next Advance, Averill Park, NY) at setting 7 for 8 minutes at 4°C. The tissue homogenate was obtained by centrifugation at 7000 rpm/15 minutes. The tissue extracts were dissolved with RIPA sample buffer (plus PPI cocktail) (Beyotime, Nantong, China). Single cell suspension was obtained by digesting the fresh lung or spleen tissues with collagenase (2mg/ml) (Sigma) and DNase I (10 µg/ml)(Roche, Mannheim, Germany) for 1h at 37°C.

Preparation of the plasma and tissue samples for Cur and THC analysis by using UHPLC/Q-TOF-MS

In brief, a stock solution of Cur and THC was prepared in 75% methanol at a concentration of 1 mg/ml. A series of working solutions ranged from 10 to 1000 ng/ml were prepared by diluting the stock solution with 75% methanol. All working solutions were stored at -20°C until use. 100 µl of plasma or lung homogenate was added with 500 µl of methanol to precipitate the proteins. After centrifugation at 15,000 rpm for 10 min at 4°C, the supernatant was transferred to a new centrifuge tube and evaporated to dryness under the gentle stream of nitrogen. The residue was reconstituted in 200 µl of mixture of water–Acetonitrile (50:50, v/v), and then 5 µl of standard working solutions and each sample was injected into the UHPLC/Q-TOF-MS instrument for analysis.

Liquid chromatography/ Mass spectrometry (LC/MS) analysis

Cur and THC were analyzed using a Waters ACQUITY™ UHPLC system coupled with a Waters SYNAPT G2-Si Q-TOF mass spectrometer (Waters, Manchester, UK). The chromatographic separation was achieved using an ACQUITY BEH C18 column (50 mm×2.1 mm i.d., 1.7 µm) at 40°C. The mobile phase consists of 0.05% aqueous formic acid (phase A) and acetonitrile containing 0.05% formic acid (phase B) at a flow-rate of 0.4 ml/min under the following gradient program: 30% B (0 - 2 min), 30% to 70% B (2 - 8 min), 70 % to 30% B (8 - 9 min), isocratic 70% B for 3 min of equilibration. Quantification analysis was performed using TOF-MRM in positive ion mode. The following MRM transitions (precursor m/z → product m/z) were monitored for quantification: m/z 369.1→285.1 for Cur and m/z 373.2→137.1 for

THC, respectively. The collision energy was set to ramp of 30 - 60 V for each MRM transition acquisition. The key operating parameters of Q-TOF MS was as follows: capillary voltage of 2.5 kV; sample cone voltage of 40 V; source temperature of 120 °C; desolvation temperature of 450 °C with a nitrogen gas flow of 900 L/h; cone gas flow of 10 L/h.

Histopathology analysis

Lung tissues sections were stained with hematoxylin and eosin (HE), periodic acid-Schiff (PAS), Masson to examine eosinophil infiltration, mucus production and collagen deposition, respectively. The number of eosinophils was counted in the fields around bronchi per lung section; the degree of mucus production was quantified by evaluating the ratio of PAS-positive area among the epithelium region; while the level of collagen deposition was quantified by measuring the percentage of positive stained area around the bronchi. The above three parameters were observed in 5-8 randomly selected fields at 1000x magnification and analyzed by using Image J software(version1.47, Media Cybernetics, Rockville, MD, USA).

Western blot

30 µg of proteins from tissue extract was resolved on 10% SDS-PAGE gel, and then the proteins were transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA). The membranes were blocked in blocking buffer and subsequently

incubated overnight at 4°C with antibodies targeting IL-4Rα (Abcam, Cambridge, UK), anti-JAK1 (Abcam), anti-pJAK1 (Try1022, Abcam), STAT6 (Abcam), pSTAT6 (Try641, Abcam), GATA3 (Cell Signaling Technology, Danvers, MA), Jagged1 (Immunoway, Plano, TX), Jagged2 (Immunoway), NICD1 (Immunoway), Notch1 (Abcam), NICD2 (Immunoway), Notch2 (Cell Signaling Technology) and β-Actin (Cell Signaling Technology). HRP-conjugated secondary antibody (Cell Signaling Technology) was then added to the membrane and detected through chemiluminescence (Millipore, Billerica, MA, USA). Protein band intensities were quantified using ImageJ Software.

Immunohistochemistry

Protein expressions of IL-4Rα, pJAK1 and pSTAT6 in lung tissues were examined by immunohistochemical staining using a modified horseradish peroxidase (HRP) technique with the DakoCytomation EnVision+System-HRP (Dako, Glostrup, Denmark). Antigen retrieval was performed with Target Retrieval Buffer (Dako). Tissue sections were then stained with rabbit anti-mouse IL-4Rα, pJAK1, pSTAT6, CD68, and CD11c polyclonal antibodies (Abcam) respectively and incubated at 4°C overnight. Species- and subtype-matched antibodies were used as negative controls (Dako). The slides were then incubated with DAKO EnVision+System-HRP (Dako) at room temperature for 30 minutes. Diaminobenzidine was used as a substrate for color development. All slides were counterstained with hematoxylin. Percentages of IL-4Rα⁺, pJAK1⁺, pSTAT6⁺, Jagged1⁺, NICD1⁺, or NICD2⁺ cells area of lung sections were

determined in 5-8 randomly selected fields per lung section for each mouse at 1000× magnification by using Image J software.

Quantitative PCR

Total RNA was extracted using mirVana™ miRNA Isolation Kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol, and 1µg of total RNA was reverse-transcribed to complementary DNA using a PrimeScript RT kit (Takara, Dalian, China). The primers for the genes (IL-4, IL-5, IL-13, IFN-γ, IL-17A, IL-33, IL-9, IL-10, GATA3, T-bet, RORc, and Foxp3) were listed in **Table E4**. Real-time quantitative PCR was performed on an ABI ViiATM7Dx Real-Time PCR System (Applied Biosystems, Foster City, CA) by using SYBR Premix Ex Taq (Takara). Relative expression of mRNAs was calculated by the $2^{-\Delta\Delta CT}$ method using β-actin as a housekeeping gene for normalization.

Table E1 Numbers of mice used in each test

Materials and Experiment	Mice in each group (number)
Nasal symptom evaluation	8
Body weight monitoring	8
Peripheral blood cell analysis	8
Histopathological staining	8
Oxidative stress analysis	5
Cytokine analysis	8
Flow cytometry experiment	4
Western blot analysis	3
Immunohistochemical staining	8
mRNA quantitative PCR	5

Table E2 Flow cytometry antibodies

Target antigen	Clone	Conjugation
CD3e	145-2C11	PE-eFluor® 610
CD4	GK1.5	APC-eFluor® 780
CD8a	53-6.7	FITC
IFNgamma	XMG1.2	APC
IL-17A	eBio17B7	PE
IL-4	11B11	PE-Cyanine7
CD25	PC61.5	PE-Cyanine7
IL-10	JES5-16E3	APC
Foxp3	FJK-16s	PE

Table E3 Summary and comparison between Cur and THC effects on modulating the inflammatory parameters

Parameters	Cur vs. Asthmatic group (% decrease)	THC vs. Asthmatic group (% decrease)	THC vs Cur <i>p</i> -value ^a
Nasal symptom (rubbing frequency)	-38.68*	-61.32*	<i>P</i> =0.024
Weight recovery ^b	3.75	6.51*	NS
blood eosinophils	-53.40	-80.84 *	NS
ELR	-46.54	-86.93 *	NS

Eos (IHC)	-54.69 *	-86.32 *	$p=0.039$
Macrophages(IHC)	-48.07 *	-64.66 *	$P=0.034$
Dendritic cells(IHC)	-14.42	-40.38 *	NS
Goblet cell hyper-production	-48.52 *	-89.60 *	$p=0.041$
Collagen deposition	-48.07 *	-54.20 *	NS
GSH (Cell lysate)	13.33	75.63 *	NS
MDA (Cell lysate)	-48.19 *	-54.69 *	NS
IL-4 (BALF)	-21.93	-70.64 *	$p=0.035$
IL-5 (BALF)	-25.97	-86.86 *	$p=0.017$
IL-13 (BALF)	-55.21 *	-62.46 *	NS
IL-17A(BALF)	-40.44	-60.10 *	NS
TH2 cells (Flow cytometry)	-37.16	-58.15 *	NS
TH17 cells (Flow cytometry)	-47.88 *	-41.75 *	NS
TC2 cells (Flow cytometry)	-66.10 *	-63.87 *	NS
TC17 cells	-44.89 *	-73.77 *	NS
IL-4Ra (WB)	-12.97 *	-39.93 *	$p<0.001$
p-Jak1 (WB)	-34.55 *	-31.71 *	NS
p-STAT6 (WB)	-43.75 *	-32.50 *	NS
GATA3 (WB)	-19.24 *	-29.27 *	$p=0.033$
Jagged1(WB)	-11.26	-37.18 *	$P=0.019$
Jagged2(WB)	-32.60 *	-55.87 *	$P=0.048$
NICD1(WB)	-23.46 *	-45.68 *	$P=0.002$
NICD2(WB)	-19.40 *	-21.23 *	NS
IL-4Ra (IHC)	-61.74 *	-84.60 *	$p=0.019$
p-Jak1 (IHC)	-57.80 *	-87.35 *	$p=0.037$
p-STAT6 (IHC)	-51.06 *	-73.29 *	$p=0.038$
Jagged1(IHC)	-47.99	-71.01 *	NS

NICD1 in epithelium (IHC)	-22.54 *	-29.41 *	NS
NICD1(IHC)	-17.06	-62.12 *	<i>P</i> =0.011
NICD2(IHC)	-51.61 *	-70.32 *	NS

a, comparison between the effect of Cur-treated group vs. THC-treated group in modulating the indicated parameter. NS, not significant.

b, weight recovery indicates the increase of weight (%).

*, indicate the change of Cur treated group vs. asthmatic group or THC treated group vs. asthmatic group is statistically significant (a p value less than 0.05).

Table E4 Primers for RT-PCR

target genes	forward primer	reverse primer
β-actin	5'-TAGGCGGACTGTTACTGAGC-3'	5'-TGCTCCAACCAACTGCTGTC-3'
IL-4	5'-GGTCTCAACCCCCAGCTAGT-3'	5'-GCCGATGATCTCTCTCAAGTGAT-3'
IL-5	5'-TCAGGGGCTAGACATACTGAAG-3'	5'-CCAAGGAACTCTTGCAGGTAAT-3'
IL-13	5'-CAGCCTCCCCGATACCAAAA-3'	5'-CTCCTCATTAGAAGGGGCCG-3'
IL-17A	5'-GGCTGACCCCTAAGAAACCC-3'	5'-AAGCAGTTTGGGACCCCTTT-3'
IFN-γ	5'-CTGCTGATGGGAGGAGATGT-3'	5'-TTTGTCAATTCGGGTGTAGTCA-3'
IL-10	5'-AGCCTTATCGGAAATGATCCAGT-3'	5'-GGCCTTGTAGACACCTTGGT-3'
IL-9	5'-ATGTTGGTGACATACATCCTTGC-3'	5'-TGACGGTGGATCATCCTTCAG-3'
IL-33	5'-ATTTCCCCGGCAAAGTTCAG-3'	5'-AACGGAGTCTCATGCAGTAGA-3'
GATA3	5'-AAGCTCAGTATCCGCTGACG-3'	5'-GTTTCCGTAGTAGGACGGGAC-3'
T-bet	5'-CGTTTCTACCCCGACCTTCC-3'	5'-ATGCTCACAGCTCGGAACTC-3'
RORγt	5'-TCCACTACGGGGTTATCACCT-3'	5'-AGTAGGCCACATTACACTGCT-3'
Foxp3	5'-AGCAGTGTGGACCGTAGATGA-3'	5'-GGCAGGGATTGGAGCACTT-3'

Figure legends

Figure S1. The appearance of around the mouse nose. The representative photo of

mice showed the appearance of bristles around the nose.

Figure S2. Food intake and the analysis of blood cells. (A) Food intake among the four groups. The bar charts show the numbers of whole blood cell (B), red blood cells (D), and platelets, as well as blood basophil percentage (C) in the four groups. Data are shown as mean $\pm$ SEM, # $p<0.05$ versus control; * $p<0.05$ ** $p<0.01$ *** $p<0.001$. Data were analyzed using a one-way analysis of variance (ANOVA) followed by Tukey-Kramer *post hoc* test for multiple comparisons.

Figure S3. The levels of IL-10⁺ Tregs in lungs. Gating of Treg (CD3⁺CD4⁺CD25⁺Foxp3⁺) cell population and the representative results of expression of IL-10 in the Treg cell population (Left) and the statistical chart (Right) of mean fluorescence intensity of IL-10 expression in Treg cells. Data are shown as mean $\pm$ SEM, # $p<0.05$ versus control; * $p<0.05$ ** $p<0.01$ *** $p<0.001$. Data were analyzed using a one-way analysis of variance (ANOVA) followed by Tukey-Kramer *post hoc* test for multiple comparisons.

Figure S4. Different Th or Tc response in the spleen tissues from asthmatic mice. Gating of CD3⁺CD4⁺ cell population (A) and the representative pictures of Th1 (CD3⁺CD4⁺IFN- γ ⁺) (B), Th2 (CD3⁺CD4⁺IL-4⁺) (C), Th17 (CD3⁺CD4⁺IL-17A⁺) (D) and Treg (CD3⁺CD4⁺CD25⁺Foxp3⁺) (E) cell population percentages were shown and the

difference of these cell subsets across the four groups were analyzed. Gating of CD3⁺CD8⁺ cell population (F) and the representative pictures of Tc1 (CD3⁺ CD8⁺ IFN- γ ⁺) (G), Tc2 (CD3⁺ CD8⁺ IL-4⁺) (H) and Tc17 (CD3⁺ CD8⁺ IL-17A⁺) (I) cell population percentages were shown and difference of these cell subsets across the four groups were analyzed. Data are shown as mean $\pm$ SEM, # p <0.05 versus control; * p <0.05 ** p <0.01 *** p <0.001. Data were analyzed using a one-way analysis of variance (ANOVA) followed by Tukey-Kramer *post hoc* test for multiple comparisons.

Figure S5. The effects of Cur and THC on the levels of mRNAs expression encoding Th1, Th2, Th17 and Treg cell related genes in lung tissues. The relative mRNA expression levels of IL-4, IL-5, IL-13, IFN- γ , IL-17A, IL-33, IL-9, IL-10, GATA3, T-bet, RORc and Foxp3 were analyzed by quantitative PCR. Data are shown as mean $\pm$ SEM, # p <0.05 versus control; * p <0.05 ** p <0.01 *** p <0.001. Data were analyzed using a one-way analysis of variance (ANOVA) followed by Tukey-Kramer *post hoc* test for multiple comparisons.