

Effects of cigarette smoke on the aggravation of ovalbumin-induced asthma and the expressions of TRPA1 and tight junctions in mice

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ABSTRACT

The occurrence of asthma is closely related to environmental factors such as cigarette smoke (CS), one of the common risk factors. Environmental stimuli have the potential to activate transient receptor potential ankyrin 1 (TRPA1) and cause or aggravate asthma. The destruction of tight junctions (TJs) between airway epithelial cells by environmental stimuli in asthma has been researched. It is worth exploring whether CS can injury TJs and aggravate asthma by activating TRPA1. The objective of this study was to investigate the aggravation of CS on ovalbumin (OVA)-induced asthma related phenotypes and TJs expression in mice, and to explore the relationship between TRPA1 and the expression of TJs protein. Female wild type (WT) C57BL/6 mice, induced by OVA, CS and OVA plus CS (OVA + CS) respectively, were used to establish a 42-day asthma model, and mice with TRPA1 knockout (TRPA1^{-/-}) were treated in the same way. This study detected the number of inflammatory cells in peripheral blood and bronchoalveolar lavage fluid (BALF), the levels of IL-4, IL-5, IL-13 in BALF, enhanced pause (Penh) of lung function, pathological changes and the gene and protein expressions of TRPA1 and TJs (including ZO-1, Occludin and Claudin-2) in lung tissues. Compared with normal saline (NS) group, WT mice in the OVA group and OVA + CS group were significantly higher in asthma related phenotypes. The WT-OVA + CS group also showed higher Penh value, levels of IL-5 and IL-13 in BALF and lung tissue injury scores when compared with the WT-OVA group and WT-CS group. However, WT-OVA + CS group mice had significantly larger number of neutrophils in BALF than the WT-OVA group, and had larger number of eosinophils in peripheral blood and higher levels of IL-4 in BALF than the WT-CS group. Meanwhile, compared with the WT-NS group, the expressions of TRPA1 and Claudin-2 in lung tissues increased in other three groups while their expressions of ZO-1 and Occludin decreased, among which, the WT-OVA + CS group showed more remarkable changes. Compared with the WT-OVA + CS group, mice in the TRPA1^{-/-}-OVA + CS showed a significant decrease in the number of inflammatory cells, levels of IL-4, IL-5 and IL-13 in BALF, Penh value and lung tissue injury score, and a downregulation of Claudin-2 expression while an upregulation of ZO-1 and Occludin expressions. In addition, the airway inflammation and injury, and the expressions of ZO-1, Occludin and Claudin-2 expressions were found with no statistic differences between TRPA1^{-/-}-OVA group and TRPA1^{-/-}-OVA + CS group. These results suggest that CS has aggravated the airway inflammation, pathological damage and destruction of TJs in airway epithelium of OVA-induced asthmatic mice, the processes of which are related to the increase of TRPA1 expression.

1. Introduction

Allergic asthma is a complex clinical heterogeneous disease characterized by reversible airflow limitation, chronic airway inflammation and airway hyperresponsiveness (Papi et al., 2018). It is highly

correlated with environmental factors such as air pollutants and allergens (Jia et al., 2020; Sakurai et al., 2018). For asthma, exposure to allergens can lead to recruitment of inflammatory cells and subsequent secretion of Th2-associated cytokines, such as IL-4, IL-5 and IL-13 (McMillan and Lloyd, 2004). Its pathological manifestations include

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epithelial and submucosal structural changes, including mucus hypersecretion, airway inflammation, airway remodeling and bronchial obstruction, which are caused by repeated airway injury, repair and regeneration (Apter, 2015). As the interface of external and internal environments, airway epithelial layer, mainly covered by airway epithelial cells, directly contacts with external environment. It is the first physical barrier to resist the invasion of pathogenic microorganisms, remove the harmful substances and maintain the airway epithelium homeostasis (Tan et al., 2019). Airway epithelia, which can be injured by infectious agents (such as virus, bacteria and fungi) and non-infectious agents (such as air pollutants and allergens), are an important part of innate immune system and play an important role in both innate and adaptive immunity of the host. The airway epithelium injury requires rapid repair and regeneration in order to restore its integrity and functions (Iosifidis et al., 2016).

Tight junctions (TJs), one of the mechanical barriers of airway epithelium, consist of transmembrane proteins including Claudins, Occludin, submembrane proteins (i.e zonula occludens [ZO-1, ZO-2, and ZO-3]) and cingulin (Hellings and Steelant, 2020; Kanda et al., 2018; Li et al., 2015). TJs most importantly function as closing the paracellular space between adjacent epithelial cells, which is directly related to the integrity of the airway epithelial barrier. When the airway epithelial layer is damaged by mechanical injury, chemical substances or biological stimulation, there will be leakage in epithelial barrier and the permeability will increase. Thus, allergens, microorganisms and their products and pollutants are more likely to pass through epithelial cells, activating the immune system and causing or aggravating allergies (Sugita et al., 2018). Cytokines such as IL-4, IL-13, IFN- γ and TNF- α could cause airway epithelial barrier dysfunction through a variety of mechanisms (Petecchia et al., 2012; Saatian et al., 2013; Soyka et al., 2012). The expressions of ZO-1 and Occludin in asthmatic patients were decreased (Hellings and Steelant, 2020; Wawrzyniak et al., 2017). Studies showed that the exposure of cigarette smoke extract (CSE) to bronchial epithelial cells could lead to airway epithelial barrier dysfunction, and down-regulate several TJs and AJs proteins simultaneously (Tatsuta et al., 2019; Zeglinski et al., 2019). However, the mechanism of the effect of cigarette exposure on TJs protein expression in asthma remains unclear.

Transient receptor potential ion (TRP) channels are a superfamily of cationic channels distributed in various cell plasma membranes, particularly those in the central and peripheral nervous systems. The expression and function of TRP channels have been confirmed not only in respiratory sensory nerve fibers, but also in respiratory non-neuronal cells (Bhattacharya et al., 2008; McGarvey et al., 2014; Szallasi et al., 2007). TRPs are the targets of many environmental toxic substances and proinflammatory agents that cause respiratory diseases. After activation, they can induce inflammatory reaction and repair process (André et al., 2009; Suresh et al., 2015). Therefore, their function in airway and lung homeostasis has aroused attention (De Logu et al., 2016). Researches have shown that numerous environmental chemical irritants (including trimellitic anhydride [TMA], PM2.5, active ingredients of tobacco smoke such as acrolein and crotonaldehyde, and House Dust Mite [HDM]) can activate TRPA1 channel and then promote the release of Calcitonin Gene Related Peptide (CGRP), Substance P(SP), Neurokinin A, etc. (Hajna et al., 2020; Li et al., 2019a,b; Lin et al., 2015; Liu et al., 2017). These substances can activate mast cells, T and B lymphocytes to produce inflammatory factors, causing bronchospasm and contraction, inducing airway inflammation and ultimately causing asthma (Dembla et al., 2016; Kanda et al., 2018; Yao et al., 2019). Previous studies have also shown that TMA or PM2.5 could aggravate OVA-induced asthma and remarkably increase the expression levels of IL-4 and IL-13, which might be related to the activation of TRPA1 channel (Li et al., 2019a,b; Liu et al., 2017). Furthermore, we studied the expression of TRPA1 in CD4⁺ T cells, and preliminarily validated the potential effect of TRPA1 in the neuro-immune interaction in airway inflammation (Li et al., 2020). Therefore, it exhibits a foreseeable

remarkable pathophysiological significance to study the influencing factors and potential mechanisms of TRPA1 (Jha et al., 2015). There were less reports on the relation of TRPA1 to the expression of TJs protein in epithelial cells (Kanda et al., 2018; Yao et al., 2019), while several studies have shown that cytokines like IL-13 are closely related to epithelial barrier (Heller et al., 2005; Holgate, 2007), which is worthy of in-depth study.

In this study, WT C57BL/6 mice were induced by OVA or CS so as to establish the 42-day asthma model. The differences of inflammatory cells number in peripheral blood and BALF, Penh of lung function, and lung tissue pathological changes in the NS group, OVA group, CS group and OVA + CS group were compared; The gene and protein expressions of TRPA1 and ZO-1, Occludin and Claudin-2 were detected. Subsequently, the differences of the above-mentioned phenotypes, and gene and protein expression levels of ZO-1, Occludin, Claudin-2 between WT and TRPA1^{-/-} mice were compared as well. This study aimed to investigate the effect of CS on OVA induced TJs expression and the aggravation of related asthma phenotypes in asthmatic mice, as well as the regulation of TRPA1.

2. Materials and methods

2.1. Animals and ethics statement

Female WT C57BL/6 mice (8–10 weeks old, 20 $\pm$ 2 g) were obtained from Beijing Weitong Lihua Laboratory Animal Technology Co. Ltd. (Beijing, China) and TRPA1^{-/-} mice (C57BL/6 background) were provided by Nanjing University of Chinese Medicine. The mice were acclimated to laboratory conditions for one week before the experiments. All animal procedures were approved by the Laboratory Animal Center of Nanjing University of Chinese Medicine (Nanjing, Jiangsu, China) and confirmed to Guide for the care and use of laboratory animals (National Academy of Sciences, 2011).

WT mice were randomly divided into four groups (n = 9/group): NS group (NS sensitizing and NS challenge), OVA group (OVA sensitizing and OVA challenge), CS group (NS sensitizing, NS challenge and CS exposure), and OVA plus CS (OVA + CS) group (OVA sensitizing, OVA challenge and CS exposure). TRPA1^{-/-} mice were randomly divided into three groups (n = 5/group), treated the same as corresponding WT groups: NS group, OVA group and OVA + CS group.

2.2. Experimental protocols of mice model

NS sensitizing and NS challenge: Mice in the NS group and CS group were treated with 200 μ L 0.9 % normal saline (NS) (including 100 μ L subcutaneously and 100 μ L intraperitoneally) on day 1 and day 8. Mice in the NS group were challenged by inhaling 0.9 %NS for 20 min once a day from day 15 to day 28 and every other day from day 30 to day 42 (Fig. 1).

OVA sensitizing: Mice in the OVA, OVA + CS group, were sensitized by 200 μ L OVA solution (100 μ g OVA with 20 μ g Al (OH)₃ being grinded and administered in 200 μ L 0.9 % NS), including 100 μ L solution subcutaneously and 100 μ L solution intraperitoneally on day 1 and day 8. (Fig. 1) (Li et al., 2020; Niu et al., 2019). Ovalbumin (OVA) was from Shanghai Bio Science and Technology Co., Ltd. (Shanghai, China) and Aluminum hydroxide (Al (OH)₃) was from Shanghai Meixing Chemical Co., Ltd. (Shanghai, China).

OVA challenge: Mice in the OVA group and the OVA + CS group were placed in closed containers (5/group) and were challenged respectively through inhaling 5% OVA solution (1 g OVA being grinded and administered in 20 mL 0.9 % NS) by ultrasonic nebulizers for 20 min once a day from day 15 to day 28 and every other day from day 30 to day 42 (Fig. 1). (M. Li et al., 2020)

CS exposure: Mice in the CS group and the OVA + CS group were subjected to continuous cigarette smoke exposure (Volume Concentration:300–400 ppm) in a Plexiglas chamber (40 $\times$ 35 $\times$ 20 cm). The

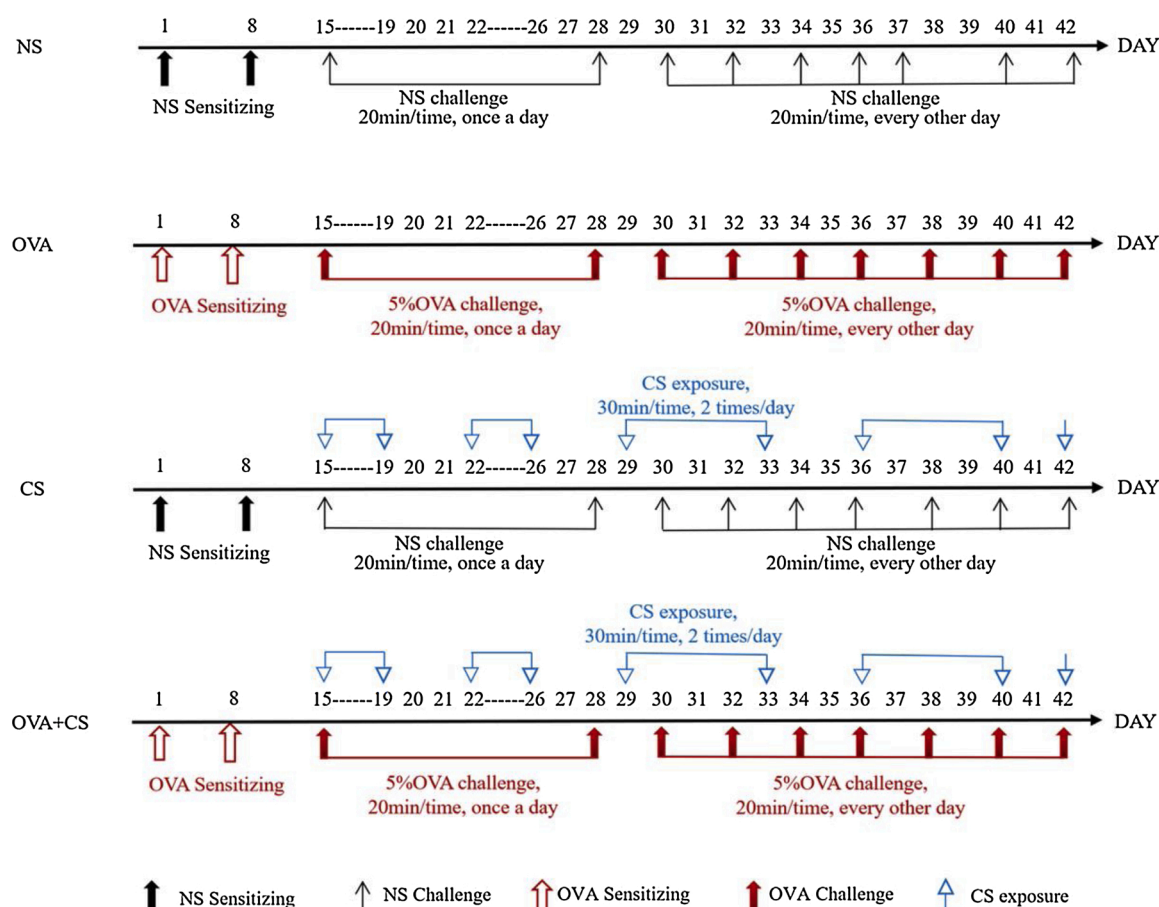


Fig. 1. Experimental protocols for the 42-day asthma model. C57BL/6 WT mice were randomly divided into four groups ($n = 9$ /group): NS group, OVA group, CS group and OVA + CS group. TRPA1^{-/-} mice were randomly divided into three groups ($n = 5$ /group): NS group, OVA group and OVA + CS group.

mice were challenged by cigarette smoke for 30 min/time, 2 times/day, 5 days/week, from day 15 to day 42 (Fig. 1). Commercial cigarettes (Double Happiness, 0.8 mg of nicotine, 10 mg of tar, and 12 mg of CO per cigarette) was from Shenzhen Tobacco Industry Co., Ltd. (Shenzhen, China).

2.3. Eosinophil count in peripheral blood

380 μ L eosinophil count liquid (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) was mixed with 20 μ L peripheral blood. Then the cell suspension was dripped into the hemocytometer, and eosinophils were counted under the microscope (Olympus, Japan) with 10 $\times$ and 20 $\times$ objective lenses within 30 min. The number of eosinophils per liter of blood was converted according to the instructions ($X \times 20 \times 10^6$). (Li et al., 2020)

2.4. BALF collection and inflammatory cell count

Mice were anesthetized and intubated through trachea, and the left lung of mice was ligated. BALF was collected by rinsing the right lung with 1.5 mL (three aliquots, 0.5 mL/time) phosphate buffered saline (PBS, pH 7.2). Then the BALF samples were centrifuged (3000 r/min) for 10 min, and the supernatant was collected and stored at -80°C . The cell-pellets were resuspended in 200 μ L of PBS and then dripped into the 10 squares of hemocytometer to count the number of total white blood cells (WBC) under 10 $\times$ and 20 $\times$ objective lenses. The total WBC count per liter of BALF was converted according to the instructions ($X \times 200 \times 10^6$). Afterwards, the samples were centrifuged (3000 r/min, 10 min), smeared, fixed and then stained by the wright's stain (Nanjing

Jiancheng Bioengineering Institute, Nanjing, China). Similarly, the percentage of eosinophils and neutrophils in BALF were calculated double-blindly using a light microscope under 10 $\times$ and 20 $\times$ objective lenses. (Li et al., 2020)

2.5. Enhanced pause (Penh)

After atomization inhalation with PBS and different concentrations of acetylcholine chloride (0, 6.25, 12.5, 25, and 50 mg/mL, ACH) respectively within 24 h after the last challenge, the lung function was tested with the noninvasive Whole body plethysmography (EMKA, France), and the Penh value reflecting airway resistance was recorded for each group. ACH was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.6. Histological assessment

The left lung tissue was fixed in 10 % formalin (Shanghai Aladdin Bio-Chem Technology Co., Ltd., Shanghai, China), dehydrated with different concentrations (80 %, 90 %, 95 %, 100 %) of ethanol (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China), and permeabilized with xylene (Shanghai Aladdin Bio-Chem Technology Co., Ltd., Shanghai, China) before being embedded with paraffin and sliced. Then the paraffin sections were dewaxed and stained with hematoxylin and eosin (HE) (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China). Images were captured under a light microscope.

Lung injury scores were determined using a semiquantitative scoring method in which lung injury was graded from 0 (normal) to 3 (severe) and was evaluated based on the following parameters: inflammatory cell

infiltration, bronchial ciliary epithelium injury (adhesion, lodging, degeneration and necrosis), mucus secretion and bronchial smooth muscle thickening. The total lung injury score (TLIS) was calculated by adding the score of each category.

2.7. Quantitative real-time polymerase chain reaction (Q-PCR) analyses

As described previously (Li et al., 2019a,b), RNA was extracted as the procedure outlined in the Trizol RNA Extraction Kit (Cat. #15596-026, Invitrogen Inc., USA), and 1 µg of RNA was used for reverse transcription according to the instructions of iScript cDNA Synthesis kit (Cat. #1708891, Bio-Rad Laboratories Inc., USA). Afterward, referring to SYBR fluorescence real-time quantitative kit (Cat. #04913850001, Roche Inc., Switzerland) procedure, the expressions of TRPA1, ZO-1, Occludin, Claudin-2 (the primer sequences are shown in Table 1) gene transcription was measured by real-time fluorescence quantitative PCR instrument (Bio-Rad Laboratories Inc., USA). The gene expression levels were calculated using $2^{-\Delta\Delta C_t}$, and the internal reference gene was GAPDH.

2.8. Western blot analyses

Total protein was also extracted from the right lung tissue, the detailed steps were as described previously (Li et al., 2019a,b). Total protein of lung tissues was extracted using the total protein extraction kit (Cat. #YWB001, Nanjing Yi-fei-xue Biotechnology Co., Ltd., China) and quantified based on BCA Protein Quantitation Kit (Cat. #DQ111-01, TransGen Biotech Inc., China). The protein samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad Laboratories Inc., USA), transferred to polyvinylidene fluoride (PVDF) membrane, sealed with 5% skim milk powder for 2 h, washed with tris buffered saline + tween-20 (TBST) for 3 times, 5 min/time. Blots were incubated with rabbit anti-TRPA1 (Cat. #ABN1009, Merck Millipore, Germany), rabbit anti-ZO-1 (Cat. #21773-1-AP, Proteintech, China), rabbit anti-Occludin (Cat. #13409-1-AP, Proteintech, China) or mouse anti-Claudin-2 antibodies (Cat. #Ag25527, Proteintech Group, Inc., China) and rabbit anti-Tubulin (Cat. #11224-1-AP, Proteintech, China) overnight at 4 °C. After incubated with secondary antibodies and washed with TBST, the PVDF membrane with the ECL solution was exposed with G: BOX chemiXR5 (Bio-Rad Laboratories Inc., USA). The results were analyzed with Image J software.

2.9. Measurement of cytokine levels in BALF

Levels of IL-4, IL-5, IL-13 in BALF were measured using enzyme-linked immunosorbent assay (ELISA) kits (Cat. # YFXEM00057, Cat. # YFXEM00790, Cat. # YFXEM00013, Nanjing Yi-fei-xue Biotechnology Co., Ltd, China) according to manufacturer's instructions.

2.10. Statistical methods

Statistical analysis was performed with GraphPad Prism 8.0 software. The data were expressed as mean and standard error of mean (mean ± SEM). Results were analyzed using one-way ANOVA analysis and Brown-Forsythe and Welch ANOVA tests, unless otherwise

indicated. P-values < 0.05 was considered as statistical difference, and P-values < 0.01 was considered as statistically significant difference.

3. Results

3.1. Effects on airway inflammation, Penh and pathology of OVA or CS-induced WT mice

The number of eosinophils in peripheral blood in the OVA + CS group and OVA group was significantly larger when compared with the NS and CS groups ($P < 0.01$) (Fig. 2A). The number of total WBC in BALF of other groups was larger than that in NS group, and the number of WBC in OVA + CS group was statistically larger than that of the OVA and CS groups ($P < 0.01$, $P < 0.05$) (Fig. 2B). The eosinophil percentages in BALF of OVA + CS group and OVA group increased significantly when compared with NS and CS groups, while the neutrophil percentages in OVA + CS group and CS group increased notably compared with the NS and OVA groups ($P < 0.01$) (Fig. 2C).

According to the experimental results, the Penh value in each group generally showed a trend of gradual increase as the ACH concentration increases. At 25 mg/mL, there was a remarkable larger Penh value in OVA + CS group than the NS and CS groups ($P < 0.05$, $P < 0.01$). At 50 mg/mL, the Penh value in OVA + CS group increased more remarkably than all other groups ($P < 0.05$, $P < 0.01$), and the value in OVA group was significantly higher than that of NS group ($P < 0.05$) (Fig. 2D).

The results showed that levels of IL-4, IL-5 and IL13 all increased in OVA group and OVA + CS group, higher than those in NS group ($P < 0.05$, $P < 0.01$). Compared with OVA group, levels of IL-5 and IL-13 in OVA + CS group were higher ($P < 0.05$) (Fig. 2E).

A normal lung morphology was observed in NS group according to the lung histopathology, while in other three model groups, there were lung injuries to various degrees. In the OVA group, there were inflammatory cell infiltration and partially exfoliated bronchial ciliary epithelium. Lung histopathology in CS group showed inflammatory cell infiltration, partial shedding of bronchial ciliary epithelium as well as thickening of tracheal wall. In OVA + CS group, it showed a more severe change of airway structure and more serious inflammatory cell infiltration with partial exfoliation of bronchial ciliary epithelium, increased mucus secretion and thickened bronchial smooth muscle, as well as severely injured bronchial mucosa (Fig. 3A). Simultaneously, compared with NS group, lung injury scores in other groups increased significantly ($P < 0.01$), and the score in OVA + CS group increased more remarkably compared with OVA group and CS group ($P < 0.05$) (Fig. 3B). In the subscores of lung injury, the scores of inflammatory cell infiltration and bronchial smooth muscle thickening in OVA + CS group increased more significantly than the OVA group ($P < 0.05$) (Fig. 3B).

3.2. The gene and protein expressions of ZO-1, Occludin and Claudin-2 in OVA or CS-induced WT mice

Compared with NS group, the expressions of ZO-1 and Occludin mRNA were downregulated while those of Claudin-2 were upregulated in lung tissue of other groups ($P < 0.05$, $P < 0.01$). Especially, the most marked change was noted in OVA + CS group when compared with the OVA group and the CS group ($P < 0.05$, $P < 0.01$) (Fig. 4A).

Compared with NS group, the expressions of ZO-1 and Occludin protein all decreased in OVA group, CS group and OVA + CS group ($P < 0.05$, $P < 0.01$). The expressions of ZO-1 and Occludin in OVA + CS group were downregulated, significantly lower than those in OVA group ($P < 0.05$), and the expression of Occludin in OVA + CS group was obviously lower than that in CS group ($P < 0.05$). In addition, the expressions of Claudin-2 in OVA group and OVA + CS group were upregulated significantly when compared with the NS group ($P < 0.05$, $P < 0.01$). The OVA + CS group also had higher level of Claudin-2 protein expression than the OVA group ($P < 0.05$) (Fig. 4[B–C]).

Table 1

The primer sequences used for qPCR.

gene	Primer sequence	
	forward	reverse
TRPA1	ACGGCTACAGCAGGGAGACT	ATGTCACTGGCTCCCTGGGT
ZO-1	GCTTTAGCGAACAGAAGGAGC	TTCATTTTCCGAGACTTCACCA
Occludin	TGAAAGTCCACCTCCTTACAGA	CCGGATAAAAGAGTACGCTGG
Claudin-2	CAACTGGTGGGCTACATCCTA	ATCCAGAGGCCCTTGGAAG
GAPDH	AAGAAGGTGGTGAAGCAGG	GAAGGTGGAAGAGTGGGAGT

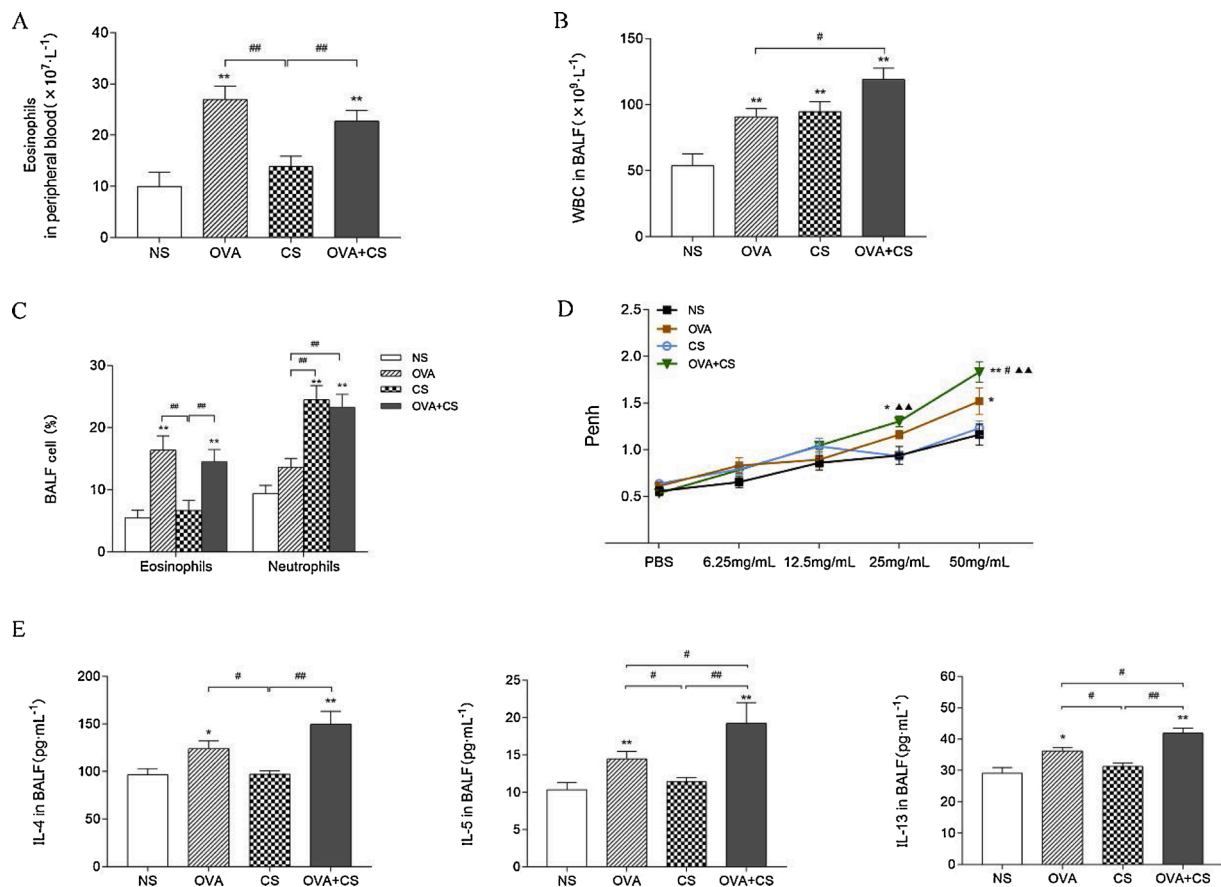


Fig. 2. The asthma phenotypes in each group. *: $P < 0.05$, **: $P < 0.01$ compared with NS group; #: $P < 0.05$, ##: $P < 0.01$ compared with other groups. (A) Eosinophil counts in peripheral blood ($n = 9$, mean $\pm$ SEM), (B) The number of total WBC in BALF, (C) Percentages of eosinophils and neutrophils in BALF ($n = 9$, mean $\pm$ SEM), (D) Changes of Penh value ($n = 9$, mean $\pm$ SEM, *: $P < 0.05$, **: $P < 0.01$ compared with NS group; #: $P < 0.05$ compared with OVA group; ▲: $P < 0.01$ compared with CS group). (E) The levels of IL-4, IL-5 and IL-13 in BALF ($n = 6$, mean $\pm$ SEM).

3.3. The gene and protein expressions of TRPA1 in OVA or CS-induced WT mice

Compared with NS group, the gene and protein expressions of TRPA1 in lung tissues of other groups were obviously higher ($P < 0.05$, $P < 0.01$). Meanwhile, those expressions in OVA + CS group were significantly upregulated compared with OVA group ($P < 0.05$, $P < 0.01$), and a remarkable increase of TRPA1 mRNA expression was detected in the OVA + CS group, which was higher than that of CS group ($P < 0.05$). (Fig. 5)

3.4. Effect on airway inflammation, Penh and pathology of WT and TRPA1^{-/-} mice

As shown in Fig. 6, compared with WT-OVA + CS group, the eosinophil counts in peripheral blood, the number of total WBC, the percentages of eosinophil and neutrophil in BALF, and Penh value at 12.5, 25 and 50 mg/mL were dramatically reduced in TRPA1^{-/-}-OVA + CS group ($P < 0.05$, $P < 0.01$). Moreover, among TRPA1^{-/-} mice groups, the differences between WT-OVA group and WT-OVA + CS group did not appear.

According to the lung histopathology, there was serious airway inflammation and injury in the WT-OVA + CS group, while TRPA1^{-/-}-OVA + CS group had mild inflammatory cell infiltration and pathological injury (Fig. 7A). The lung injury of WT-OVA + CS group was more serious than that of WT-OVA group, while the difference between TRPA1^{-/-}-OVA + CS and TRPA1^{-/-}-OVA groups became smaller (Fig. 7A). Correspondingly, compared with WT mice, the lung injury

score and subscores of TRPA1^{-/-} mice decreased significantly ($P < 0.05$, $P < 0.01$) (Fig. 7[B–C]).

3.5. The gene and protein expressions of ZO-1, Occludin, Claudin-2 in WT and TRPA1^{-/-} mice

Compared with WT-OVA + CS group, the TRPA1^{-/-}-OVA + CS group had much higher levels of ZO-1 and Occludin protein and gene expressions, as well as lower level of Claudin-2 gene and protein expression ($P < 0.05$, $P < 0.01$). In WT-OVA + CS group, the protein expressions of ZO-1, Occludin and ZO-1 mRNA were downregulated obviously compared with WT-OVA group, while the mRNA and protein expressions of Claudin-2 were significantly upregulated ($P < 0.05$, $P < 0.01$), whereas the TRPA1^{-/-} mice did not show the similar difference (Fig. 8).

4. Discussion

Asthma is one of the chronic allergic inflammatory diseases which is associated with repeated or continuous exposure to allergens. The most common immune response in asthma is Type 2 immunity (T2 response), named after Type 2 helper T lymphocyte (Th2 cells). Asthma is mostly associated with cell recruitment and mediator release driven by Th2 and ILC2s (Type 2 innate lymphoid cells), involving mast cells, eosinophils, basophils and macrophages (Holgate, 2007). After being activated by environmental pollutants, cytokines released by bronchial epithelial cells can stimulate Th2 cells and ILC2s to produce IL-4, IL-5, IL-13 and other Type 2 cytokines which have been proved to have a core role in

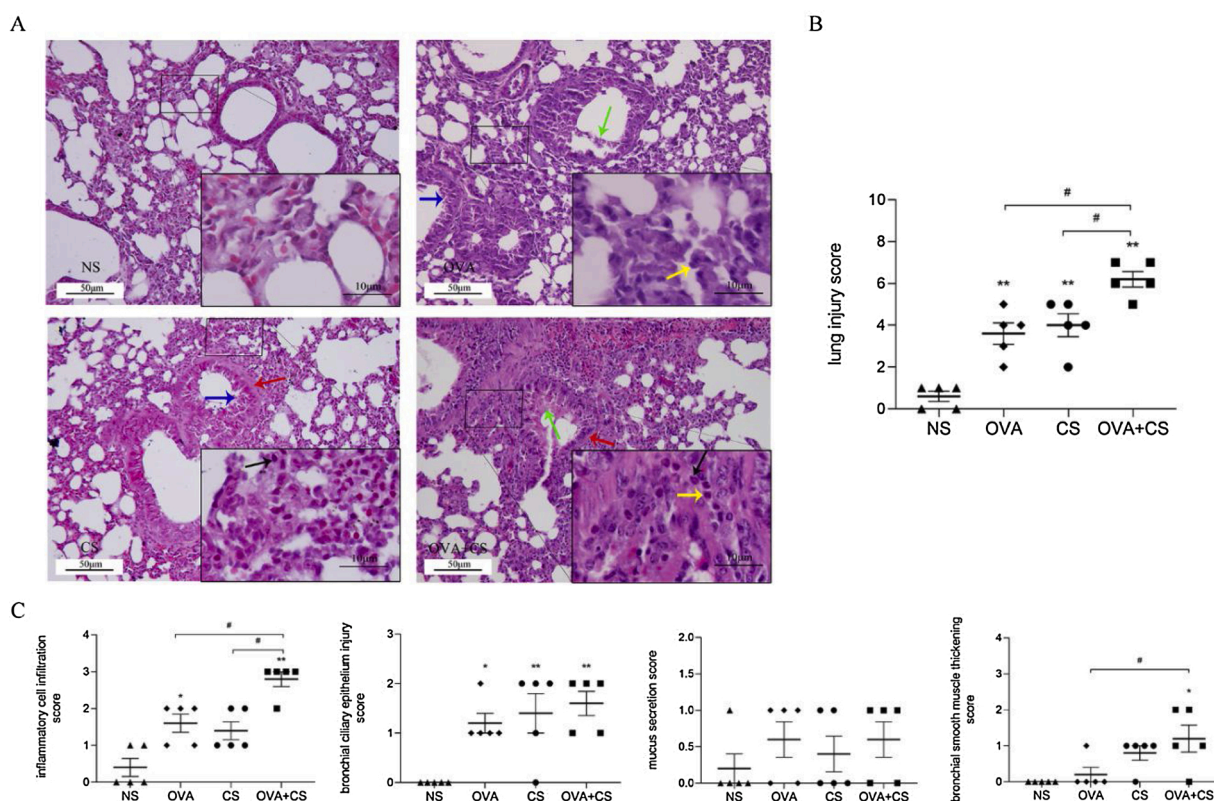


Fig. 3. Changes of lung histopathology in OVA or CS-challenged wild-type mice. (A) Lung histopathological changes (left lung tissue) stained with HE (scale bars 50 μ m, the inset scale bars 10 μ m). Green arrow: sticky bronchial secretion; blue arrow: bronchial mucosa damage; red arrow: airway epithelium stratification; black arrow: neutrophil infiltration; yellow arrow: eosinophil infiltration. (B) The semiquantitative score of lung injury. (C) The subscores of lung injury. (n = 5/group, mean $\pm$ SEM. *: P < 0.05, **: P < 0.01 compared with NS group; #: P < 0.05, ##: P < 0.01 compared with the other group.).

initiating and maintaining allergic airway responses in asthma (Akdis et al., 2020; Caminati et al., 2018; Fahy, 2015). IL-4 is the major cytokine which promotes the differentiation of initial T lymphocytes into Th2 cells, drives IgE and IgG1 synthesis by B cells, and promotes the expression of adhesion molecules (Lambrecht et al., 2019). IL-5 is responsible for the activation and recruitment of eosinophils and mast cells, while IL-13 induces goblet cell hyperplasia, mucus hypersecretion and airway hyperresponsiveness (Fahy, 2015; Zhu et al., 1999). These characteristic immune cells and cytokines can cause an immune response resulting in eosinophil accumulation and activation in the airway, mucus hypersecretion and airway hyperresponsiveness, thereby driving pathophysiology of asthma (Bruggemann et al., 2017; Hearn et al., 2020; Li et al., 2019a,b). OVA sensitization and challenge can induce a typical Type 2 immune model (Gold et al., 2015; Li et al., 2020; Russjan and Kaczynska, 2018). In this study, the number of eosinophils in peripheral blood and BALF significantly increased in OVA group, and the levels of IL-4, IL-5 and IL-13 in BALF also remarkably improved. The lung histopathology result showed numerous inflammatory cell infiltration, cilia lodging and shedding, and mucus secretion.

Exposure to allergens or environmental pollutants can damage epithelial structure and function, which in turn exacerbates exposure of the immune system to allergens such as primary ones. Continuous and repeated exposure can aggravate chronic allergic inflammation and form a vicious cycle of repetitive airway epithelial injury, repair and regeneration (Girodet et al., 2011; Holgate, 2007; Mauad et al., 2007). Cigarette smoke is one of the most common inhaled respiratory irritants. Chronic exposure to CS could prolong the survival time of neutrophils and increase the number of neutrophils in lung tissues (Polosa and Thomson, 2013; Zhou et al., 2020; Zou et al., 2020). CS could also promote the activation of bronchial epithelial cells, the metaplasia of goblet cells, airway inflammation and airway remodeling (Yang et al.,

2020). In this study, the levels of eosinophils, neutrophils and Type 2 cytokines increased in OVA + CS group. The level of neutrophil percentage in BALF of OVA + CS group was significantly higher than that in OVA group. While the increase of eosinophil percentage in OVA + CS group was not significant compared with that of the OVA group, it might be related to the significantly higher level of neutrophil percentage. At the same time, the lung injury in OVA + CS group was more serious. HE staining showed massive inflammatory cell infiltration, partial exfoliation of ciliary epithelium, severe thickening of bronchial smooth muscle and severe injury of bronchial mucosa. The results indicated that CS exposure could aggravate airway allergic inflammation and injury in OVA-induced asthmatic mice.

Airway epithelial barrier maintains epithelial homeostasis mainly by maintaining normal structure and function, and the intact structure is the basis of epithelial homeostasis. It was shown that in the early stage of inflammation, selective induction of autophagy in asthmatic epithelial cells and dendritic cells could maintain the regeneration ability of airway progenitor cells, thus effectively repairing the structure of injured epithelial cells and preventing acute inflammation from developing into chronic one (Li et al., 2019a,b). However, continuous injury and repair of epithelial cells may lead to lung structural changes and airway remodeling, which can progress to chronic inflammation stage of asthma. Hence, it is particularly important to prevent or reverse deleterious remodeling (Holgate, 2007; Mauad et al., 2007). As the basic components of TJs structure and function in epithelial barrier, ZO protein and Occludin mainly function as closing the paracellular space between adjacent epithelial cells, and preventing the invasion of allergens, microorganisms and pollutants. Claudins are a large family of tetramer transmembrane proteins, which can promote both the barrier formation and leakage. Claudin-2 is a pore-forming protein and a leaky-type tight junction protein. It plays an important role in the

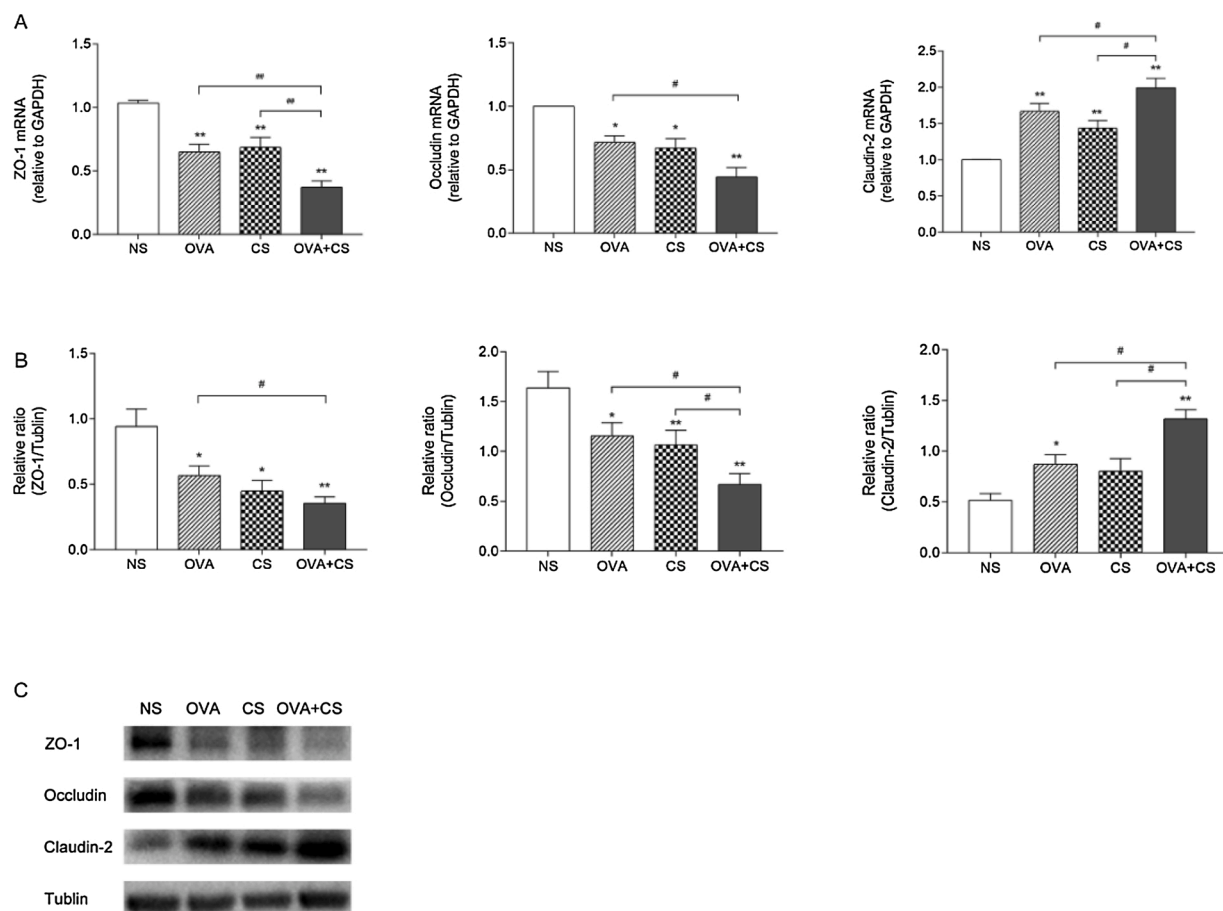


Fig. 4. The gene and protein expressions of ZO-1, Occludin and Claudin-2 in lung tissue ($n = 3$, mean $\pm$ SEM, *: $P < 0.05$, **: $P < 0.01$ compared with NS group; #: $P < 0.05$, ##: $P < 0.01$ compared with other groups). (A) The gene expressions of ZO-1, Occludin and Claudin-2 (relative to GAPDH), (B) The protein expressions of ZO-1, Occludin and Claudin-2 (relative to Tubulin), (C) Gel images showing protein expression.

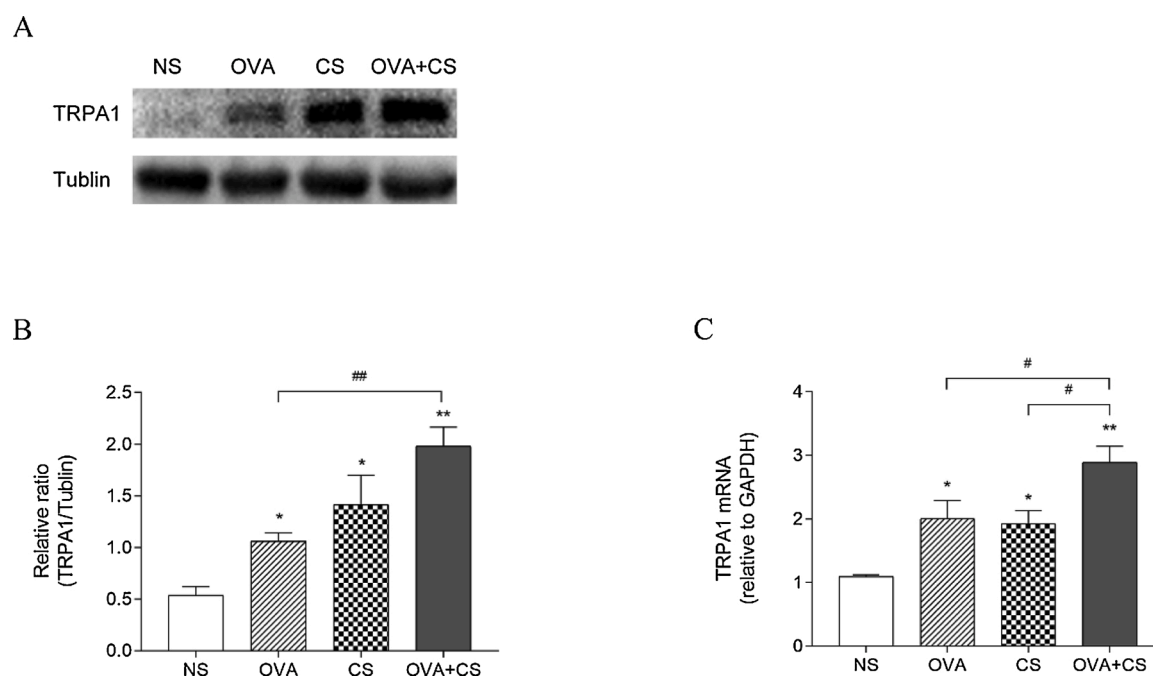


Fig. 5. The gene and protein expressions of TRPA1 in lung tissues ($n = 3$, mean $\pm$ SEM, *: $P < 0.05$, **: $P < 0.01$ compared with NS group; #: $P < 0.05$, ##: $P < 0.01$ compared with other groups). (A) Gel images showing protein expression, (B) The protein expressions of TRPA1 (the relative ratio of protein), (C) The gene expressions of TRPA1 (relative to GAPDH).

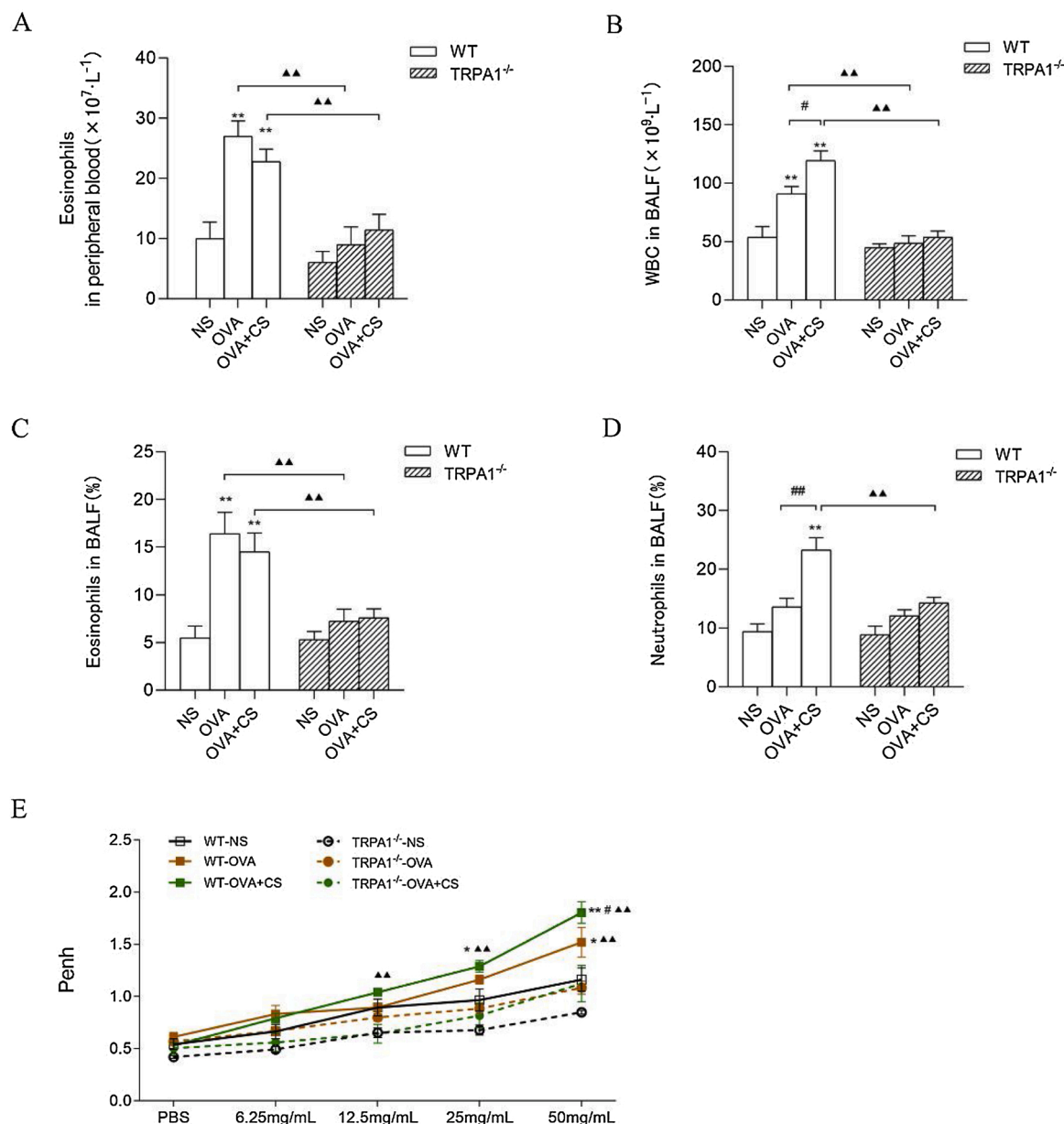


Fig. 6. The asthma phenotypes in each group of WT mice and TRPA1^{-/-} mice (9/group in WT, 5/group in TRPA1^{-/-}, mean $\pm$ SEM. *: $P < 0.05$, **: $P < 0.01$ compared with WT-NS group; #: $P < 0.05$, ##: $P < 0.01$ compared with WT-OVA group; ▲: $P < 0.05$, ▲▲: $P < 0.01$ compared with TRPA1^{-/-} mice). (A) Eosinophil counts in peripheral blood. (B) The numbers of total WBC in BALF. (C) Percentages of eosinophils in BALF. (D) Percentages of neutrophils in BALF. (E) Changes of Penh value.

formation of cell bypass pore channel, cation permeability, ion size selectivity and water transportation (Georas and Rezaee, 2014; Han et al., 2020; Okada et al., 2020). Studies have shown that IL-13 could promote permeability and delay repair of colon epithelial cell monolayers by stimulating apoptosis and increasing expression of the pore-forming tight junction protein Claudin-2 (Heller et al., 2005; Rosen et al., 2013). Therefore, the decreased expressions of ZO-1 and Occludin indicate epithelial barrier injury or leakage, while the increased expression of Claudin-2 can also lead to the same result. It was noted that smoke and cigarette smoke extract (CSE) could inhibit the expressions of Occludin, E-cadherin and ZO-1, damage the integrity of epithelial barrier, increase the inflammation and epithelium injury, and destroy the host defense of airway epithelium (Tatsuta et al., 2019). Besides, allergens such as HDM extracts could destroy the expressions of Occludin, ZO-1 and E-cadherin (Jia et al., 2020; Yao et al., 2019). In this study, compared with NS group, the levels of IL-4, IL-5, IL-13 in OVA

group and OVA + CS group significantly increased, the expressions of ZO-1 and Occludin in lung tissues were downregulated, and the gene and protein expressions of Claudin-2 were upregulated. Furthermore, changes of OVA + CS group were more significant than those in OVA group, indicating that CS aggravated the injuries of TJs in asthma airway epithelium.

Studies have shown that TRPs channels are involved in the regulation of epithelial barrier function. For example, TRPA1 is involved in TDI-induced pulmonary epithelial barrier dysfunction and asthma airway inflammation (Yao et al., 2019). Capsaicin and compound 2 could activate TRPA1, induce Ca^{2+} influx and increase TJs permeability (Kanda et al., 2018). Previous experiments proved that the asthma aggravated by environmental particulate pollutants (like PM2.5, TMA) might be related to the activation of TRPA1 channel (Li et al., 2019a,b; Li et al., 2020; Liu et al., 2017). This study showed that the gene and protein expressions of TRPA1 in OVA + CS group were significantly

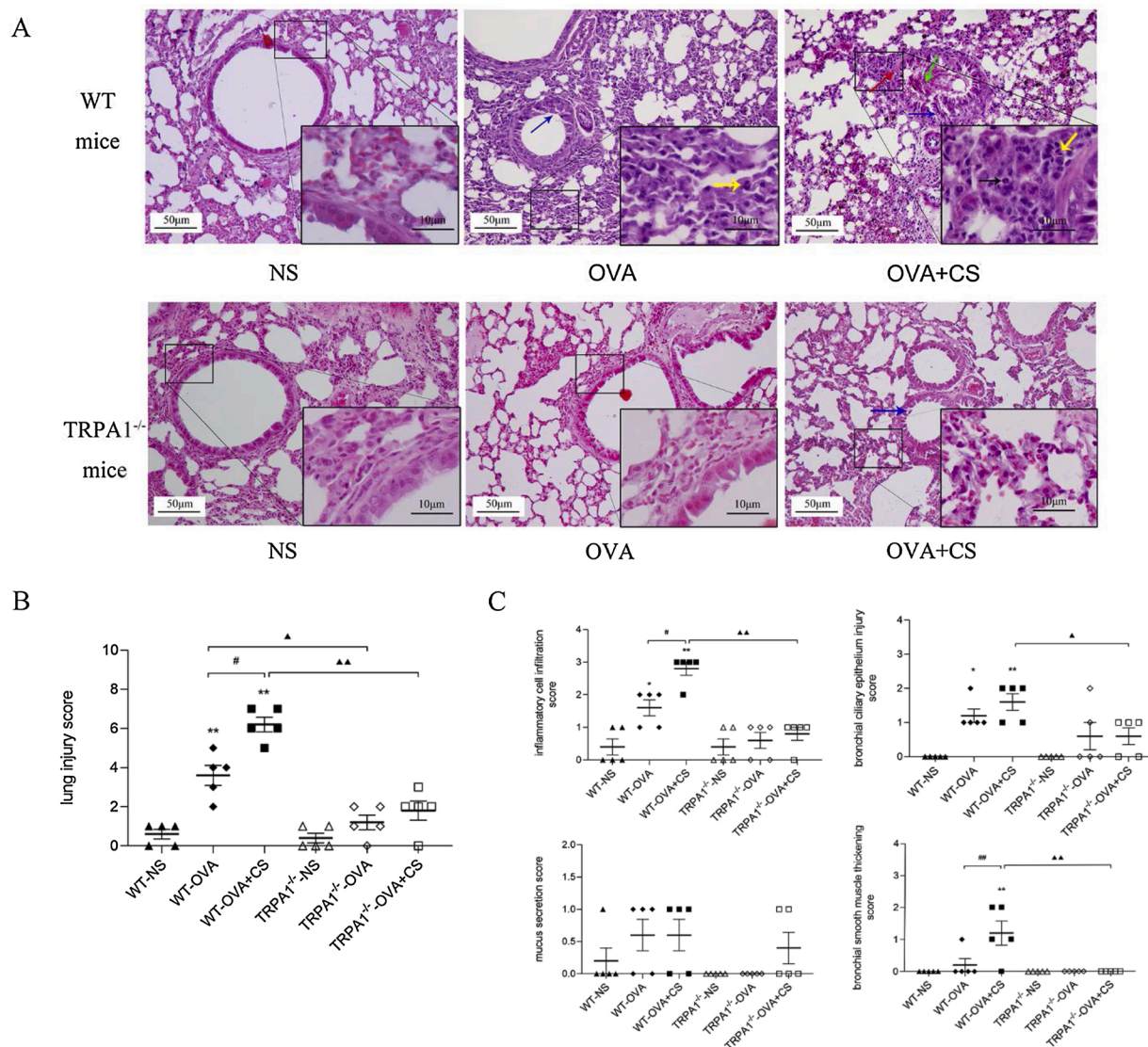


Fig. 7. Changes of lung histopathology in WT mice and TRPA1^{-/-} mice. (A) Lung histopathological changes (left lung tissue) stained with HE (scale bars 50 μ m, the inset scale bars 10 μ m). Green arrow: sticky bronchial secretions; blue arrow: bronchial mucosa injury; red arrow: airway epithelium stratification; black arrow: neutrophil infiltration; yellow arrow: eosinophil infiltration. (B) The semiquantitative scores of lung injury. (C) The subscores of lung injury. (n = 5/group, mean $\pm$ SEM. *: P < 0.05, **: P < 0.01 compared with WT-NS group; #: P < 0.05, ##: P < 0.01 compared with WT-OVA group; ▲: P < 0.05, ▲▲: P < 0.01 compared with TRPA1^{-/-} mice.).

higher than those in the OVA group, which suggested that CS could activate TRPA1 channel more strikingly in asthmatic mice. To further explore whether CS aggravating OVA-induced asthma was related to the destruction of TJs and the regulatory effect of TRPA1, TRPA1^{-/-} mice and WT mice induced by OVA or OVA + CS were compared. According to the results, the asthma related phenotypes in TRPA1^{-/-}-OVA + CS group significantly decreased compared with WT-OVA + CS group. HE staining in lung tissues of TRPA1^{-/-} mice indicated a mild inflammatory cell infiltration. The gene and protein expressions of ZO-1 and Occludin increased remarkably, while Claudin-2 expression in lung tissues decreased significantly in TRPA1^{-/-}-OVA + CS group compared with WT-OVA + CS group. In addition, the differences of airway inflammation and injury, as well as the expressions of ZO-1, Occludin and Claudin-2 between OVA group and OVA + CS group in TRPA1^{-/-} mice were significantly reduced, indicating that the exacerbation of asthma phenotypes and the epithelial barrier injury induced by CS could be eliminated by TRPA1 gene knockout. In other words, CS could aggravate the destruction of epithelial barrier function in asthma, which might be related to the enhanced activation of TRPA1.

In conclusion, CS exposure could aggravate OVA-induced asthma airway inflammation, airway hyperresponsiveness and pathological injury, and decrease the expressions of ZO-1 and Occludin, as well as increase the expression of Claudin-2 in lung tissues. It reveals that CS might aggravate asthma allergic inflammatory response and airway injury by destroying TJs between airway epithelial cells, and the activation of TRPA1 was linked to these changes. However, the mechanism of TRPA1 regulation of airway epithelial barrier needs to be further investigated. It may be significant in improving the epithelial barrier function of asthmatic individuals and reducing the vicious cycle of epithelial damage and repair.

CRedit authorship contribution statement

Yu-bo Sun: Methodology, Formal analysis, Investigation, Writing - original draft. **Mo Liu:** Investigation, Formal analysis. **Xin-sheng Fan:** Project administration, Conceptualization, Methodology, Writing - review & editing, Supervision, Funding acquisition. **Li-ping Zhou:** Investigation, Validation. **Meng-wen Li:** Resources, Investigation.

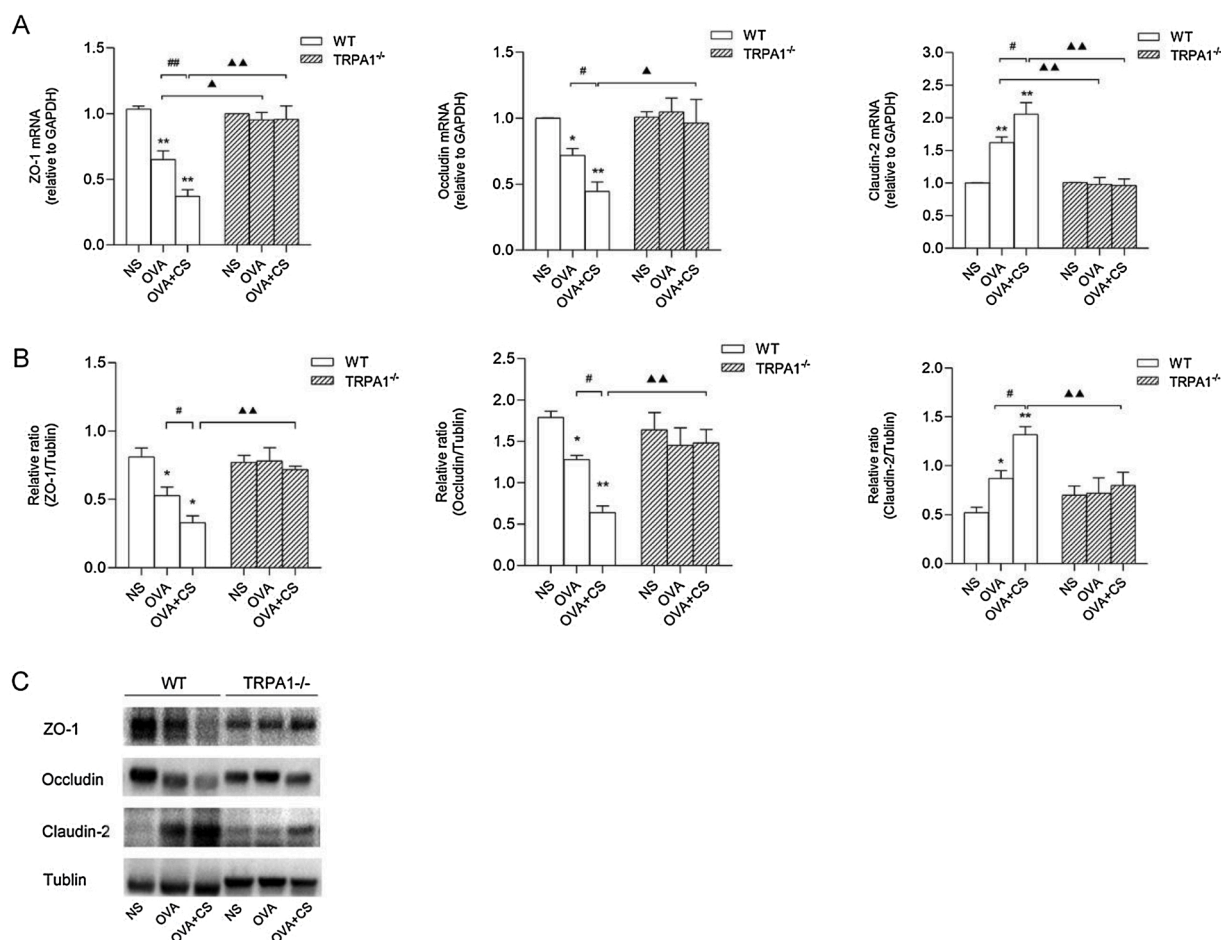


Fig. 8. Protein expressions of ZO-1, Occludin and Claudin-2 in lung tissues of WT and TRPA1^{-/-} mice (n = 3, mean ± SEM, *: P < 0.05, **: P < 0.01 compared with WT-NS group; #: P < 0.05, ##: P < 0.01 compared with WT-OVA group; ▲: P < 0.05, ▲▲: P < 0.01 compared with TRPA1^{-/-} mice.). (A) The gene expressions of ZO-1, Occludin and Claudin-2 (relative to GAPDH). (B) The protein expressions of ZO-1, Occludin and Claudin-2 (the relative ratio of protein). (C) Gel images showing protein expressions.

Fang-yuan Hu: Investigation. **Qin-fei Yue:** Investigation, Formal analysis. **Yi-ming Zhang:** Investigation.

Declaration of Competing Interest

The authors report no declarations of interest.

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