International Immunopharmacology 69 (2019) 159–168

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Exacerbating effects of trimellitic anhydride in ovalbumin-induced asthmatic mice and the gene and protein expressions of TRPA1, TRPV1, TRPV2 in lung tissue

Mengwen Li[a], Xinsheng Fan[a,*], Leilei Ji[b], Yuhao Fan[a], Li Xu[b]

[a] School of Basic Medicine, Nanjing University of Chinese Medicine, Nanjing, China
[b] School of Pharmacy, Nanjing University of Chinese Medicine, Nanjing, China

ARTICLE INFO

Keywords:
Asthma
Trimellitic anhydride
TRPA1
TRPV1
TRPV2

ABSTRACT

With the increasing morbidity and mortality of asthma, asthma aggravated by environmental pollution has drawn more attention. This study investigated the exacerbating effects of trimellitic anhydride (TMA), a typical pollutant, in ovalbumin (OVA)-induced asthmatic mice and the gene and protein expressions of TRPA1, V1, V2 in lung tissue. Female BALB/c mice were respectively administered for 42 days as follow: sensitized and challenged with OVA, sensitized and challenged with TMA, sensitized with OVA and challenged with OVA plus TMA, as well as sensitized and challenged with OVA plus TMA. 24 h after the last challenge, the changes in airway resistance (RI) and lung dynamic compliance (Cdyn) were tested. The levels of the inflammatory cells in blood and bronchoalveolar lavage fluid (BALF) were determined. The gene and protein expressions of TRPA1, V1, V2 in lung tissue were examined, and levels of interleukin (IL)-4, −13, substance P (SP), prostaglandin D_2 (PGD_2), nerve growth factor (NGF) in BALF and the supernatant of lung homogenate were measured. The results indicated that OVA plus TMA significantly increased the amount of inflammatory cells in blood and BALF, enhanced RI while decreased Cdyn, and aggravated lung injury. Increased gene and protein expressions of TRPA1, V1, V2 in lung tissue, level of IL-4 in the supernatant of lung homogenate, levels of IL-13, SP, PGD_2, NGF in BALF and the supernatant of lung homogenate were observed. It was suggested that exacerbating effects of TMA in OVA-induced asthma might be related to the regulation of TRPA1, V1, V2 and relevant neurokines.

1. Introduction

Asthma is recognized as one of the common pulmonary diseases and public health issues with an increasing global prevalence [1,2]. The pathological features of asthma manifest as enhanced airway hyperresponsiveness (AHR), reversible airway remodeling and chronic airway inflammation, which can lead to dyspnea, chest tightness and recurrent cough [3]. These pathophysiological changes are closely associated with elevated allergen-specific type 2 T helper (Th2) cytokines, including interleukin (IL)-4, -13. Th2 cytokines are involved in eosinophil maturation, infiltration, and activation, as well as IgE and mucus production, which eventually induce the asthmatic responses [4]. Especially, with global environment and industrial pollution worsening, the mortality and morbidity of asthma are increasing for the exposure to various environmental pollutants [5,6].

Trimellitic anhydride (TMA, $C_9H_4O_5$), as a low molecular weight reactive chemical and a typical pollutant, is commonly detected in chemicals, printing and dyeing working environment [7]. The lung disease caused by TMA has become an increasingly serious public health problem [8,9]. It has been shown that TMA could provoke typical Th2 immune response [9–11]. The release of pro-inflammatory TH2 cytokine IL-4 into the BALF was also significantly increased by TMA [9]. The levels of Th2 cytokines such as IL-4, IL-13 in local lymph nodes from the mice sensitized/challenged by TMA showed significant increases [9,10]. The chemical TMA-induced Th2-biased response might be diverted during the induction period by exogenous administration of the Th2 cytokine antagonist, particularly anti-IL-4 antibody [11]. However, it was less reported that the exacerbating effects of TMA on asthma. We hypothesize that TMA, as one of the environmental pollutants, might have exacerbating effects on ovalbumin-induced asthma.

Transient receptor potential (TRP) channel is nonselective cation channels that sense a vast array of chemical and physical stimuli, which is located mostly on the plasma membrane of sensory nerve cells and

* Corresponding author.
E-mail address: fanxsh126@126.com (X. Fan).

https://doi.org/10.1016/j.intimp.2019.01.038
Received 9 February 2018; Received in revised form 11 January 2019; Accepted 27 January 2019
Available online 01 February 2019

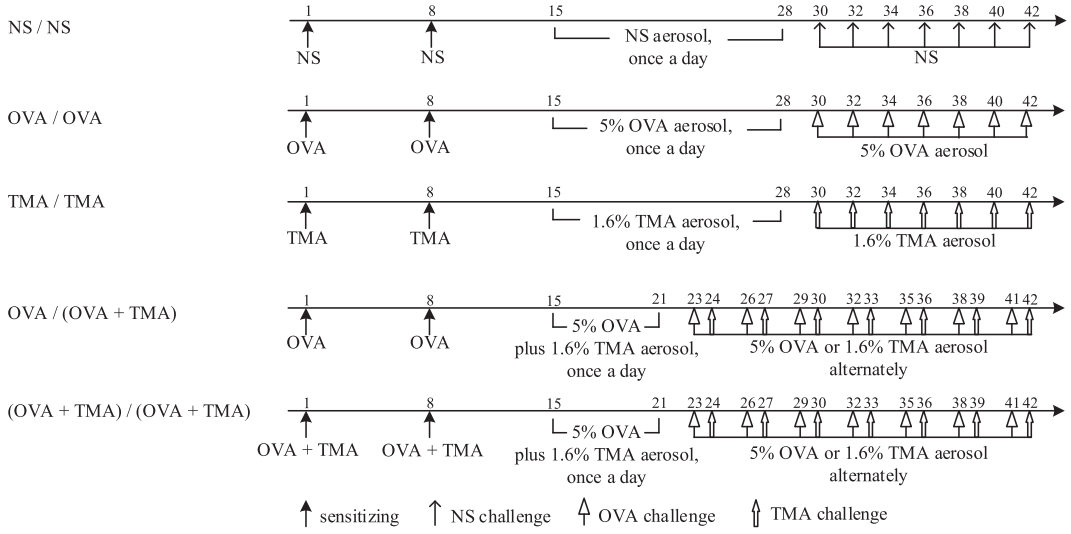

Fig. 1. Experimental protocols for the 42-day asthma model. BALB/c mice were randomly divided into five groups (n = 12/group): (1) NS/NS, (2) OVA/OVA, (3) TMA/TMA, (4) OVA/(OVA + TMA), (5) (OVA + TMA)/(OVA + TMA).

other cell types [12]. As TRP family members, TRPA1, V1 and V2 play an indispensable role in the development of asthma [13–15]. Several neurogenic inflammation mediator substance P (SP) [16], prostaglandin D2 (PGD_2) [17] and nerve growth factor (NGF) [18] are closely associated with airway inflammation. In our previous study, the OVA plus PM2.5 asthma model was established and the results indicated that pollutant PM2.5 possibly exacerbates asthma via regulating TRPA1, V1 and the relevant neurokines [19]. Therefore, it was speculated that the TRPs channels might play a similar role in exacerbating effects of TMA on asthma.

To verify the above hypotheses, a 42-day asthmatic mice model induced by OVA plus TMA was established. In this study, the changes in airway resistance (RI) and lung dynamic compliance (Cdyn) in response to acetylcholine chloride (ACH) were tested. The levels of the inflammatory cells in blood and bronchoalveolar lavage fluid (BALF) were determined. Pulmonary histopathological changes were observed. The gene and protein expressions of TRPA1, V1, V2 in lung tissue were examined, and levels of IL-4, IL-13, SP, PGD_2, NGF in BALF and the supernatant of lung homogenate were measured. Based on the above results, the exacerbated effects of TMA in OVA-induced asthmatic mice model were evaluated and the potential mechanism of TMA exacerbating asthma associated with TRPs regulation was discussed.

2. Method

2.1. Chemicals

Trimellitic anhydride (TMA) was from Sigma-Aldrich, Inc. (Missouri, USA) and acetone, olive oil were from Shanghai Experimental Reagent Co., Ltd. (Shanghai, China). 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. (China).

2.2. Animals and ethics statement

Female BALB/c mice (8–12 weeks old, 20 ± 2 g) were obtained from the Comparative Medicine Centre of Yangzhou University (Yangzhou, Jiangsu, China) with a certification (No. SCXK (Su) 2012-0004). 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).

2.3. Mouse model of asthma

Mice were randomly divided into five groups (n = 12/group): (1) normal control (NS sensitization/challenge, NS/NS); (2) OVA sensitization/challenge (OVA/OVA); (3) TMA sensitization/challenge (TMA/TMA); (4) OVA sensitization/OVA plus TMA challenge (OVA/(OVA + TMA)); (5) OVA plus TMA sensitization/challenge ((OVA + TMA)/(OVA + TMA)). The model replication method was listed as follows:

Sensitization: Mice in all groups were given sensitization on day 1 and day 8. NS/NS group was sensitized by 100 μL 0.9% NS subcutaneously and intraperitoneally. TMA/TMA group received an intradermal injection of total 60 μL TMA solution (35%, w/v) at two sites separately. Mice in the OVA/OVA group and OVA/(OVA + TMA) group were treated with 200 μL OVA solution (100 μg OVA with 1 mg Al $(OH)_3$) subcutaneously and intraperitoneally [17]. The (OVA + TMA)/(OVA + TMA) model group was injected with 100 μL OVA solution intraperitoneally and 30 μL of TMA solution intradermally. The TMA was suspended in AOO solution (acetone: olive oil = 4:1) to prepare 35% TMA solution [20].

Challenge: Mice in the NS/NS group, the OVA/OVA group and the TMA/TMA group were challenged respectively through inhaling 0.9% NS, 5% OVA solution and 1.6% TMA solution for 20 min from day 15 to day 28 and every other day from day 30 to day 42. Comparatively, mice in the OVA/(OVA + TMA) group and the (OVA + TMA)/(OVA + TMA) group were challenged by 5% OVA from day 15 to day 21 once a day, then challenged by 5% OVA on day 23, 26, 29, 32, 35, 38, 41 and 1.6% TMA on day 24, 27, 30, 33, 36, 39, 42. The detailed timelines were illustrated in Fig. 1.

All mice were anesthetized with 5% chloral hydrate (10 mL/kg) 24 h after the last challenge for the subsequent collection of blood, BALF and lung tissue.

2.4. Eosinophil count in peripheral blood

380 μL eosinophils count liquid (Nanjing Jiancheng Bioengineering Institute, China) and 20 μL peripheral blood were mixed evenly. The blood is diluted with eosinophils count liquid and eosinophils were stained red via the phenol method, while red blood cells and other white blood cells were broken or dissolved. Then the diluted cell suspension was dropped into the blood cell count plate to count the

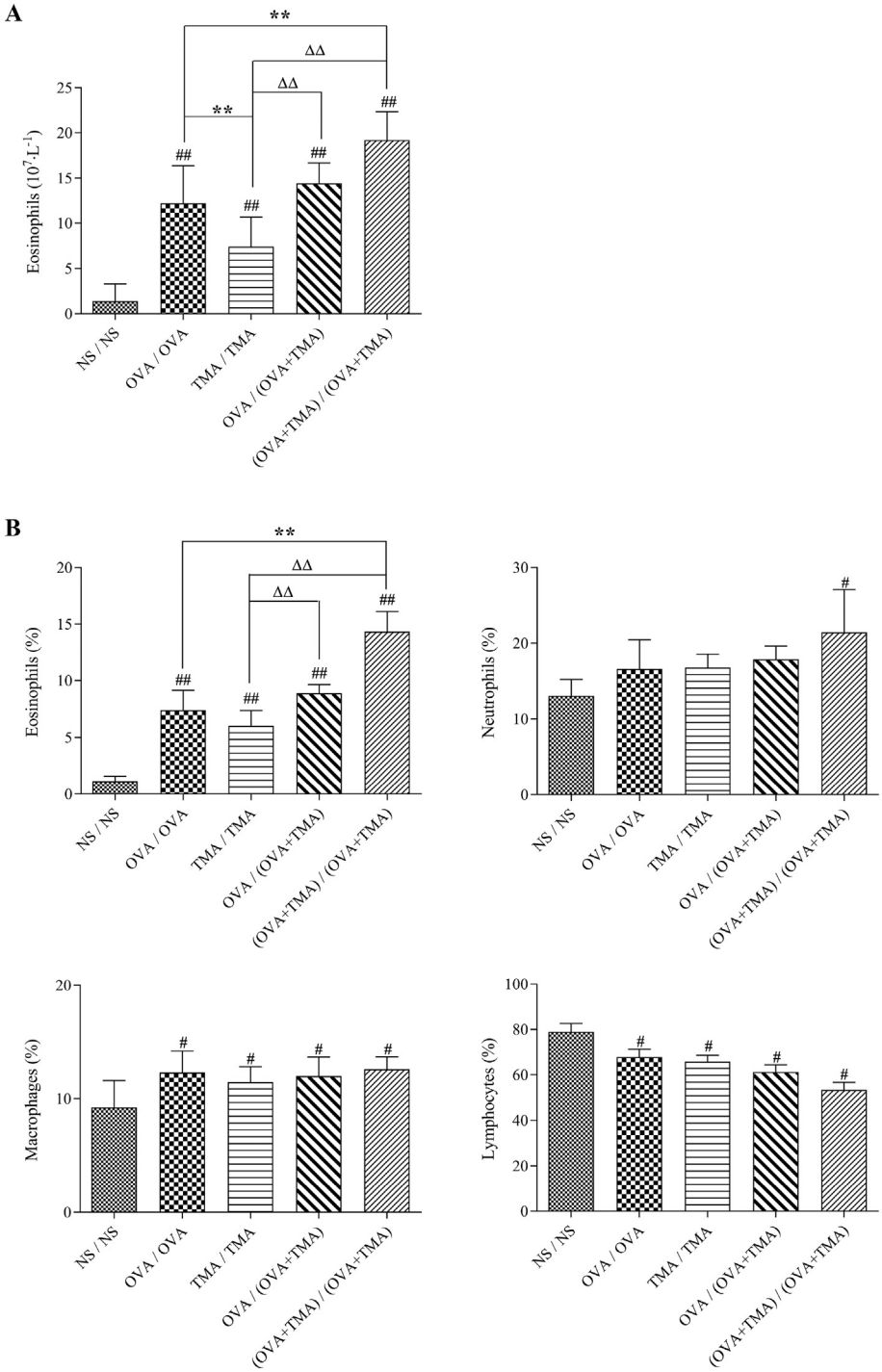

Fig. 2. Inflammatory cells in blood and BALF. (A) Eosinophil counts in peripheral blood ($n = 10$, means ± SD). (B) Percentage of inflammatory cells in BALF ($n = 6$, means ± SD). $^{\#}$: $P < 0.05$, $^{\#\#}$: $P < 0.01$ compared with NS/NS group; **: $P < 0.01$ compared with OVA/OVA group;$^{\triangle\triangle}$: $P < 0.01$ compared with TMA/TMA group.

number of eosinophils in 10 squares in a hemocytometer with 10× and 20× objective lenses. The number of eosinophils per liter of blood was converted according to the instructions ($X \times 20 \times 10^6$).

2.5. BALF collection and inflammatory cell counts

Mice were anesthetized and tracheally intubated. Bronchoalveolar lavage fluid was performed with three aliquots of 0.5 mL phosphate buffered saline (PBS, pH 7.2) withdrawn three times each. The recovery rate of fluid was 75–85%. BALF samples were centrifuged (3000 r/min) for 10 min, and the supernatant was harvested and stored at −80 °C. The pelleted cells were resuspended in 200 μL of PBS, smeared, fixed and stained by the wright's stain (Nanjing Jiancheng Bioengineering Institute, China). The percentages of different leukocytes in BALF were counted double-blindly using a light microscope (Olympus, Japan) with 10× and 20× objective lenses (each slide counts twice, 200 cells per count).

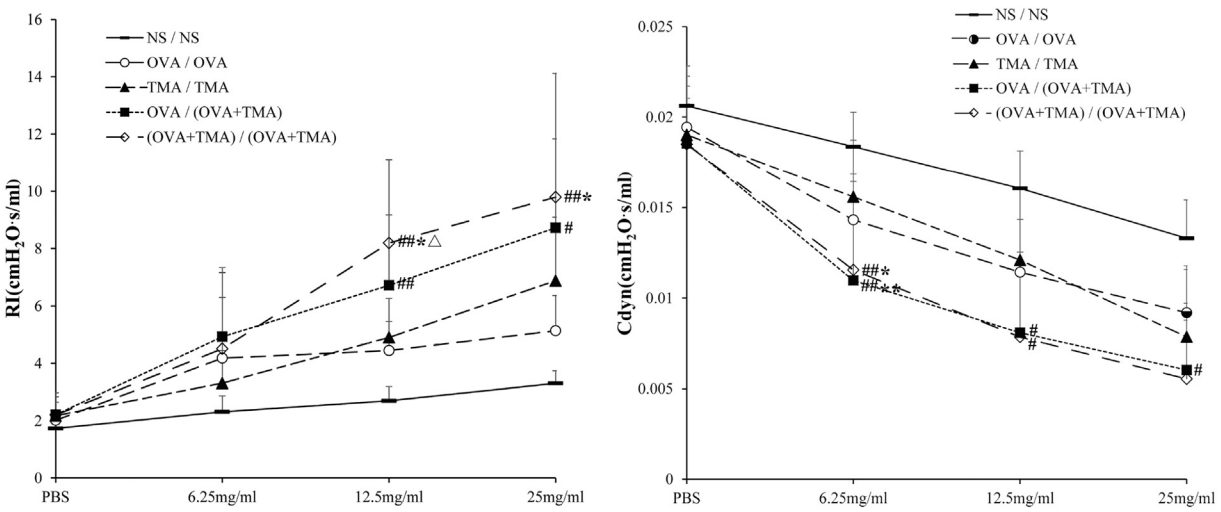

Fig. 3. Changes of RI and Cdyn ($n = 6$, means ± SD). (A) Airway resistance (RI). (B) Dynamic lung compliance (Cdyn). $^{\#}$: $P < 0.05$, $^{\#\#}$: $P < 0.01$ compared with NS/NS group; *: $P < 0.05$, **: $P < 0.01$ compared with OVA/OVA group; $^{\triangle}$: $P < 0.05$ compared with TMA/TMA group.

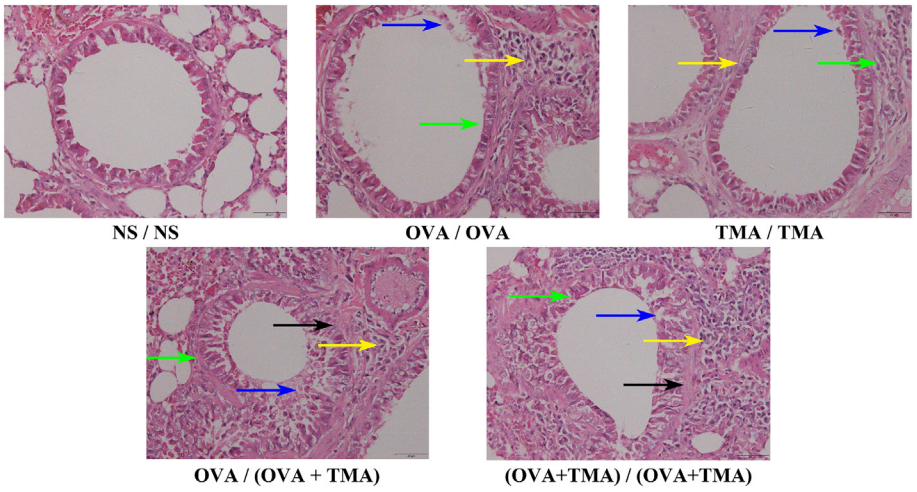

Fig. 4. Pulmonary histopathological changes stained with HE (40×). Green arrow: inflammatory cell infiltration; yellow arrow: ciliated adhesion, degeneration, necrosis; blue arrow: ciliated epithelial shedding; black arrow: airway epithelium stratification. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.6. Airway responsiveness determination

24 h after the last challenge, airway responsiveness was analyzed with acetylcholine chloride (ACH) stimulation of restrained mice by a Buxco FinePointe™ RC system (Buxco Inc., USA), which was performed as described previously [10]. After the injection of 5% chloral hydrate (10 mL/kg) intraperitoneally, mice were connected to a ventilator through tracheal intubation. The airway resistance (RI) and dynamic lung compliance (Cdyn) in response to different concentrations of ACH (0, 6.25, 12.5, 25 mg/mL) were recorded. Here, RI shows the change in air flow of airway in experimental animals and Cdyn mainly reflects the change of airway pressure.

2.7. Histological assessment

Histological assessment was performed as described previously [21]. The right lung tissues were fixed in 10% formalin and embedded in paraffin. Paraffin sections were stained with hematoxylin and eosin and inspected under a light microscope.

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

Quantitative real-time polymerase chain reaction was performed as described previously [22]. After taking about 50 mg of left lung tissue and adding 500 μL of TRIzol into it, RNA was extracted as the procedure outlined in the TRIzol RNA Extraction Kit (Invitrogen Inc., USA). The extracted RNA was stored at −80 °C. The concentration and purity of the RNA were tested with Nanodrop (Thermo Inc., USA), and 1 μg of RNA was used for reverse transcription according to the instructions of reverse transcription kit (TransGen Biotech Inc., China). Afterward, referring to SYBR fluorescence real-time quantitative kit (Roche Inc., Switzerland) procedure, the expressions of TRPA1, V1, V2 gene transcription was measured by real-time fluorescence quantitative PCR instrument. A total of 40 cycles of amplification were carried out under the following conditions: maintenance phase, 50 °C for 2 min, 95 °C for 10 min; cycle phase, 95 °C for 15 s, and 60 °C for 1 min. The gene expression levels were calculated using $2^{-\triangle\triangle Ct}$, and the internal reference gene was GAPDH. The control sample was defined as 1 to calculate the remaining groups of gene expression differences. The primers used were as follow: mouse TRPA1, forward primer ACGGCT ACAGCAGGGAGACT, reverse primer ATGTCAGTGGCTCCCTGGGT; mouse TRPV1, forward primer CATTGCTCTCATGGGCGAG, reverse primer AGTCATCCTTGCCTTCCGG; mouse TRPV2, forward primer ATAGAGCAGGAAGCTGTGGT, reverse primer TCGTCCACCCTCCACCT TAG; mouse GAPDH, forward primer GGTGAAGGTCGGTGTGAAC, reverse primer TCGCTCCTGGAAGATGGTG.

2.9. Western blot analyses

Histological assessment was performed as described previously

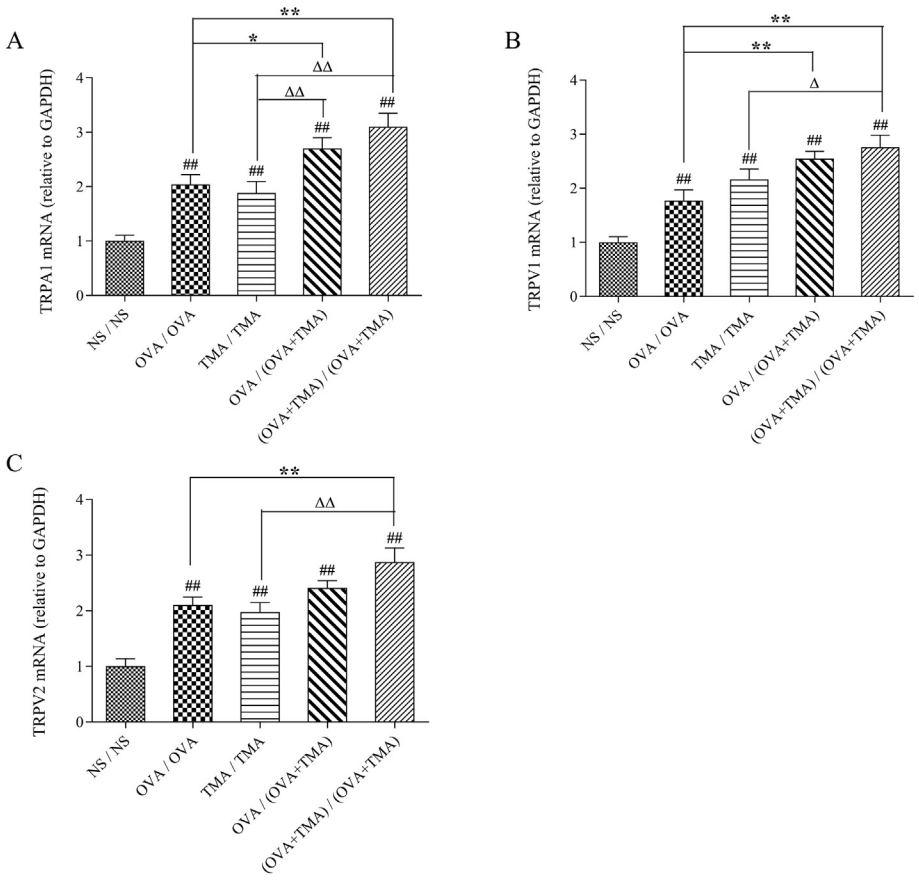

Fig. 5. The gene expressions of TRPA1, TRPV1, and TRPV2 in lung tissue ($n = 3$, means ± SD). (A) TRPA1 mRNA (relative to GAPDH), (B) TRPV1 mRNA (relative to GAPDH), (C) TRPV2mRNA (relative to GAPDH). $^{\#\#}$: $P < 0.01$ compared with NS/NS group; *: $P < 0.05$, **: $P < 0.01$ compared with OVA/OVA group; $^{\triangle}$: $P < 0.05$, $^{\triangle\triangle}$: $P < 0.01$compared with TMA/TMA group.

[23]. Total protein was also extracted from the left lung tissue, quantified using the Bradford method and diluted in denaturing lysis buffer. The samples were incubated at 95–100 °C for 5 min. Then, samples containing 50 μg proteins each were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad Laboratories Inc., USA). After electrophoresis, proteins were transferred to polyvinylidene fluoride (PVDF) membrane, blocked for 2 h in 5% skim milk powder blocking buffer and washed with phosphate buffered saline + tween-20 (PBST) 4 times. Blots were incubated with mouse anti-TRPA1, anti-TRPV1 or anti-TRPV2 antibodies (ABcam Inc., England) and GAPDH antibodies (CST Inc., USA) overnight at 4 °C. After washing with PBST four times, blots were incubated with secondary antibodies for 2 h at room temperature. The membranes were washed with PBST four times, incubated with developing a solution for 5 min at room temperature, and exposed with G: BOX chemiXR5 (Bio- Rad Laboratories Inc., USA). The results were analyzed with Gel-Pro32 software (Media Cybernetics Inc., USA).

2.10. Measurement of cytokine and neurokine levels in BALF

Levels of IL-4, IL-13, SP, PGD_2, and NGF in BALF were measured using enzyme-linked immunosorbent assay (ELISA) kits (Shanghai Yunhan Biological Technology Co., Ltd., China) according to manufacturer's instructions.

2.11. Measurement of cytokine and neurokinin levels in the supernatant of pulmonary homogenate

The lung homogenate was obtained following the previous method with appropriate adjustment [24]. The lung tissue was collected, ground and homogenized in pre-cooled saline (w:v 1:9), followed by centrifugation at 3000 rpm for 10 min. The supernatant was harvested and stored at −80 °C. The levels of IL-4, IL-13, SP, PGD_2, and NGF in the supernatant of pulmonary homogenate were measured using enzyme-linked immunosorbent assay (ELISA) kits (Shanghai Yunhan Biological Technology Co., Ltd., China) according to manufacturer's instructions.

2.12. Statistical method

Data were expressed as means ± standard deviation (means ± SD). Results were analyzed using one-way ANOVA analysis of variance followed by the Tukey post hoc test, unless otherwise indicated. *P*-values < 0.05 was considered statistical difference, and *P*-values < 0.01 was significant statistical difference.

3. Results

3.1. Effect on inflammatory cell counts

The amount of eosinophils in peripheral blood of each group was counted. The amounts in the other four groups were significantly increased ($P < 0.01$) compared with NS/NS group. There was also a significant increase of eosinophils in the (OVA + TMA)/(OVA + TMA) group ($P < 0.01$) compared with OVA/OVA group. Besides, the amounts of eosinophils in OVA/(OVA + TMA) group and (OVA + TMA)/(OVA + TMA) group were significantly increased with statistical significance ($P < 0.01$) compared with TMA/TMA group (Fig. 2-A).

Similarly, there was a significant increase of eosinophils percentage in other groups compared with NS/NS group ($P < 0.01$) in BALF. Besides, the higher eosinophils percentage in OVA/(OVA + TMA) group and (OVA + TMA)/(OVA + TMA) group were obtained compared with TMA/TMA group ($P < 0.01$), and the higher eosinophils

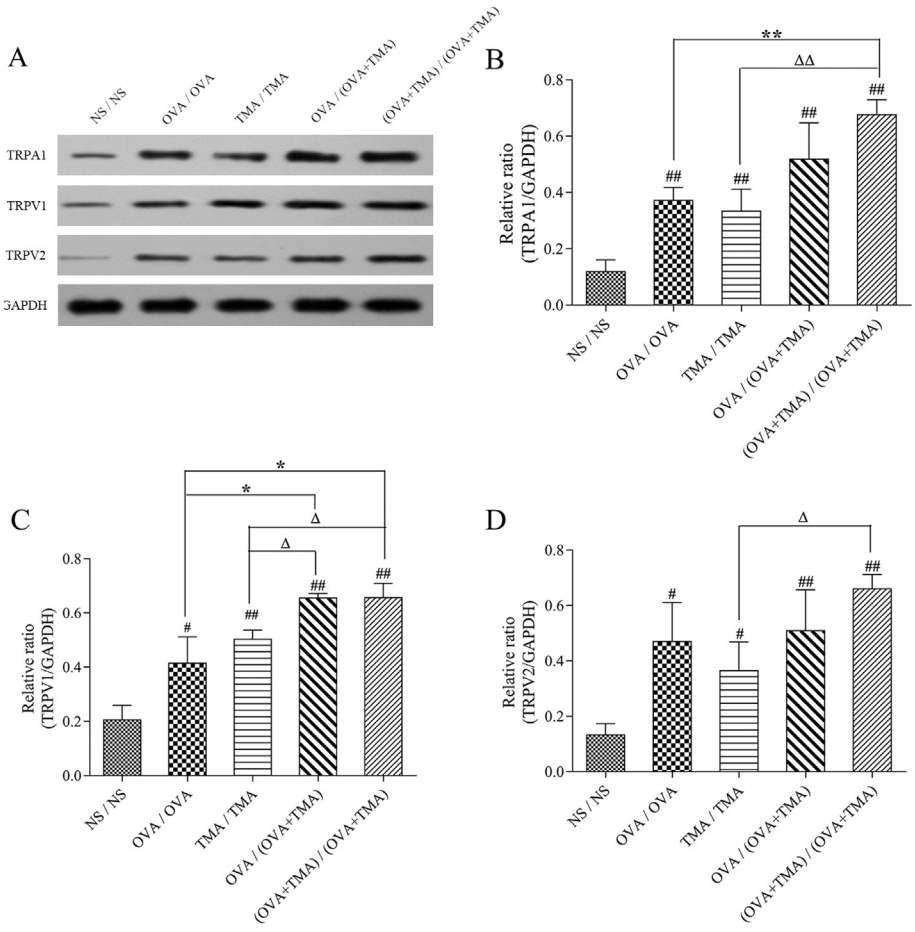

Fig. 6. Protein expressions levels of TRPA1, TRPV1 and TRPV2 in lung tissue ($n = 3$, means ± SD). (A) Gel images showing protein expression, (B) the relative ratio of TRPA1, (C) the relative ratio of TRPV1, (D) the relative ratio of TRPV2. $^{\#}$: $P < 0.05$, $^{\#\#}$: $P < 0.01$ compared with NS/NS group; *: $P < 0.05$, **: $P < 0.01$ compared with OVA/OVA group; $^{\triangle}$: $P < 0.05$, $^{\triangle\triangle}$: $P < 0.01$compared with TMA/TMA group.

percentage in (OVA + TMA)/(OVA + TMA) group was seen compared with OVA/OVA group ($P < 0.01$). The percentage of neutrophils in (OVA + TMA)/(OVA + TMA) group was increased compared with NS/NS group ($P < 0.05$). The percentages of macrophages were increased ($P < 0.05$) and the percentages of lymphocytes were relatively decreased ($P < 0.05$) in other four groups compared with NS/NS group (Fig. 2-B).

3.2. Effect on airway responsiveness

Airway responsiveness of each group after exposure to different concentrations of ACH was determined by measuring changes of RI and Cdyn (Fig. 3-A). The experimental results showed that, with the increase of ACH concentration, RI value of each group was increased, particularly in (OVA + TMA)/(OVA + TMA) group. At the ACH concentration of 12.5 mg/mL and 25 mg/mL, RI values of OVA/(OVA + TMA) group and (OVA + TMA)/(OVA + TMA) group were significantly increased compared with NS/NS group ($P < 0.05$, $P < 0.01$), and a significant increase was observed in (OVA + TMA)/(OVA + TMA) group compared with OVA/OVA group ($P < 0.05$). Compared with TMA/TMA group, RI value of OVA + TMA)/(OVA + TMA) group was significantly increased at 12.5 mg/mL ACH ($P < 0.05$).

The Cdyn values in each group were decreased with the increase of ACH concentration (Fig. 3-B). Compared with NS/NS group, the Cdyn values in OVA/(OVA + TMA) were significantly decreased at the different concentrations of ACH ($P < 0.05$, $P < 0.01$), and which in (OVA + TMA)/(OVA + TMA) group significantly were decreased for the concentration of 6.25 mg/mL and 12.5 mg/mL ($P < 0.05$, $P < 0.01$). Compared with OVA/OVA group, Cdyn values of in OVA/(OVA + TMA) and (OVA + TMA)/(OVA + TMA) group were significantly decreased at 6.25 mg/mL ($P < 0.05$, $P < 0.01$).

3.3. Changes of lung histopathology

The sections of lung tissues were prepared, subjected to hematoxylin, eosin staining and observed. NS/NS group appeared the normal lung morphologies. Other four model groups showed various degrees of lung damage compared with NS/NS group. More severe changes of airway structure were observed in the OVA/(OVA + TMA) group and (OVA + TMA)/(OVA + TMA) group, showing the more serious inflammatory cell infiltration, bronchial epithelial shedding, cilia adhesion, degeneration and necrosis, as well as moderate airway epithelium stratification in lung tissues. Representative images are shown in Fig. 4.

3.4. Gene expressions of TRPA1, TRPV1, and TRPV2

The gene expressions of TRPs in lung tissue were detected using Q-PCR. The expressions of TRPAl, TRPV1 and TRPV2 in lung tissue in other groups were significantly higher than that in NS/NS group $P < 0.01$). Also, the gene expressions of TRPAl, TRPV1 and TRPV2 in (OVA + TMA)/(OVA + TMA) group were y increased ($P < 0.05$, $P < 0.01$) compared with OVA/OVA group and TMA/TMA group. The gene expressions of TRPAl and TRPV1 in OVA/(OVA + TMA) were significantly increased compared with OVA/OVA group ($P < 0.05$, $P < 0.01$). Higher gene expression of TRPA1 was also observed compared with TMA/TMA group ($P < 0.01$) (Fig. 5).

3.5. Protein expressions TRPA1, TRPV1, and TRPV2

The protein expressions of TRPA1, TRPV1 and TRPV2 were determined by western blot. The protein expressions of TRPAl, TRPV1 and

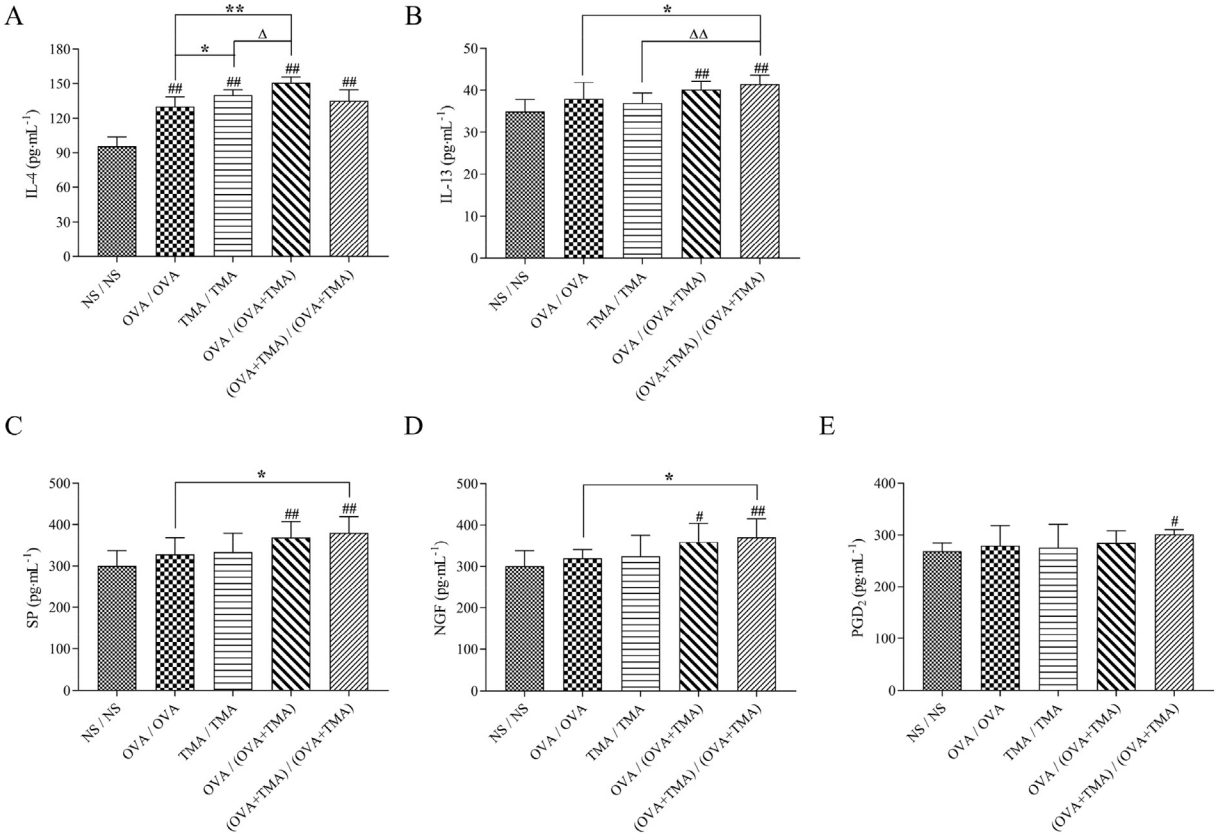

Fig. 7. The levels of IL-4, IL-13, SP, NGF and PGD_2 in BALF ($n = 6$, means ± SD). (A) IL-4 level, (B) IL-13 level, (C) SP level (D) NGF level, (E) PGD_2 level. $^{\#}$: $P < 0.05$, $^{\#\#}$: $P < 0.01$ compared with NS/NS group; *: $P < 0.05$ compared with OVA/OVA group; $^{\triangle}$: $P < 0.05$, $^{\triangle\triangle}$: $P < 0.01$compared with TMA/TMA group. Statistical analysis was performed using one-way ANOVA with Fisher's LSD post hoc test.

TRPV2 in lung tissue in other four groups were increased compared with NS/NS group ($P < 0.05$, $P < 0.01$). Particularly, expression of TRPA1 in (OVA + TMA)/(OVA + TMA) group was significantly increased ($P < 0.01$) compared with OVA/OVA group and TMA/TMA group. Besides, OVA/(OVA + TMA) and (OVA + TMA)/(OVA + TMA) groups showed higher expression of TRPV1 compared with OVA/OVA group and TMA/TMA group ($P < 0.05$). Similarly, TRPV2 protein expression in the (OVA + TMA)/(OVA + TMA) group was higher ($P < 0.05$) than that in TMA/TMA group (Fig. 6).

3.6. IL-4, IL-13, SP, NGF, PGD_2 in BALF and the supernatant of pulmonary homogenate

Levels of IL-4, IL-13, SP, NGF and PGD_2 in BALF were determined by ELISA kits. The levels of IL-4 in OVA/OVA and TMA/TMA group were increased compared with NS/NS group ($P < 0.01$). The levels of IL-4, IL-13, SP, and NGF were increased in OVA/(OVA + TMA) and (OVA + TMA)/(OVA + TMA) groups compared with NS/NS group ($P < 0.05$, $P < 0.01$). The levels of IL-4 in OVA/(OVA + TMA) groups were increased compared with OVA/OVA and TMA/TMA group ($P < 0.05$, $P < 0.01$). Besides, the level of IL-13 in (OVA + TMA)/(OVA + TMA) group was increased compared with OVA/OVA and TMA/TMA group ($P < 0.05$, $P < 0.01$).The levels of SP and NGF in (OVA + TMA)/(OVA + TMA) group were significantly increased compared with OVA/OVA group ($P < 0.05$) (Fig. 7).

Levels of IL-4, IL-13, SP, NGF and PGD2 in the supernatant of pulmonary homogenate were also determined by ELISA kits. The levels of IL-4, NGF and PGD_2 were increased in OVA/OVA group compared with NS/NS group ($P < 0.05$, $P < 0.01$). A statistical difference of IL-4, PGD_2 were observed in TMA/TMA group ($P < 0.05$, $P < 0.01$), compared with NS/NS group. The levels of IL-4, IL-13, SP, NGF and PGD_2 in OVA/(OVA + TMA) group and (OVA + TMA)/(OVA + TMA) group were significantly increased compared with NS/NS group ($P < 0.01$). (OVA + TMA)/(OVA + TMA) group showed higher levels of L-4, IL-13, SP, NGF and PGD_2 compared with OVA/OVA group ($P < 0.05$, $P < 0.01$). And the levels of IL-13, SP, NGF in (OVA + TMA)/(OVA + TMA) group were increased compared with TMA/TMA group ($P < 0.05$, $P < 0.01$). The level of SP in OVA/(OVA + TMA) group was increased ($P < 0.05$) compared with TMA/TMA group (Fig. 8).

4. Discussion

Asthma is an inflammatory disease regulated by the T helper (Th) cells, manifested as bronchial hyperresponsiveness, mucus overproduction, airway wall remodeling and airway narrowing. The Th1/Th2 imbalance is responsible for the development of allergic asthma [25], especially Th2 is thought to play a central role in initiating and sustaining asthma responses [26]. The Th2 cells is activated, and the secreted characteristic cytokines such as IL-4, IL-13 contribute to hallmarks of asthma, including airway eosinophilia, increased mucus production, and development of airway hyper-responsiveness [27–29]. TMA, known as a typical low-molecular-weight chemical sensitizer, could induce the typical Th2 response. Long-term airway contact with TMA induced persistent airway irritation and breathing pattern changes [30]. In this study, we established a 42-day BALB/c mice model of asthma induced by OVA plus TMA. The data demonstrated that TMA exacerbated asthma phenotypes, including a higher amount of inflammatory cells in blood and BALF, more apparent changes of RI and Cdyn in response to ACH, and severe lung histopathology. Additionally, the mice treated by OVA plus TMA showed higher gene and protein expressions of TRPA1, V1, and V2, as well as the levels of Th2 cytokine

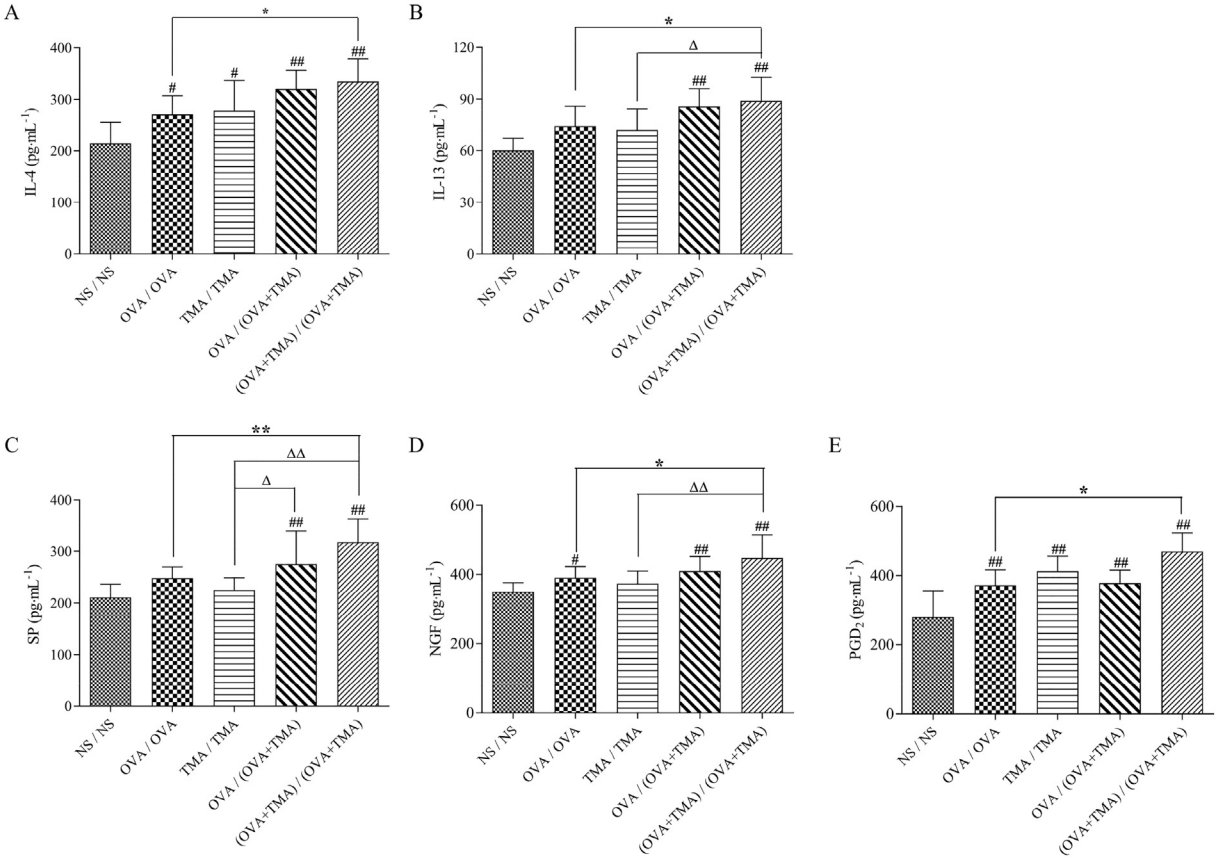

Fig. 8. The levels of IL-4, IL-13, SP, NGF and PGD_2 in the supernatant of pulmonary homogenate ($n = 5$, means ± SD). (A) IL-4 level, (B) IL-13 level, (C) SP level (D) NGF level, (E) PGD_2 level. $^{\#}$: $P < 0.05$, $^{\#\#}$: $P < 0.01$ compared with NS/NS group; *: $P < 0.05$, **: $P < 0.01$ compared with OVA/OVA group; $^{\triangle}$: $P < 0.05$, $^{\triangle\triangle}$: $P < 0.01$compared with TMA/TMA group. Statistical analysis was performed using one-way ANOVA with Fisher's LSD post hoc test.

IL-13, SP, PDG2 and NGF.

The eosinophil is believed as an important inflammatory effector cell in asthma [31]. Our study showed that the OVA plus TMA increased the amount of inflammatory cells, especially eosinophils level in blood and BALF. Meanwhile, RI was increased while Cdyn was decreased in the mice treated by OVA plus TMA. The histopathological assessment in our investigation showed TMA resulted in more severe lung injury in the asthmatic mice model, manifesting as apparent inflammatory cell infiltration, bronchial epithelial shedding, ciliary adhesion lodging, degeneration, necrosis, and airway epithelial stratification. All the above results suggested that TMA aggravated asthma phenotype.

In this study, based on the BALB/c mice model sensitized/challenged by OVA, TMA, as a combined sensitizer or activator, was added during the modeling process. Our data suggests that Th2 cytokines play a role in aggravated TMA induced asthma. IL-13 is thought can induce AHR airway inflammation, goblet cell metaplasia and subepithelial fibrosis [32–35]. The levels of IL-13 in BALF and the supernatant of lung homogenate were both significantly increased in the mice sensitized/challenged by OVA plus TMA. IL-4 has been shown essential in the initiation of allergic airway responses and humoral responses [36]. The level of IL-4 in BALF was increased in OVA/OVA + TMA, and which in the supernatant of lung homogenate was increased in (OVA + TMA)/(OVA + TMA), compared with in OVA/OVA. Confusingly, there was no significant increase in level of IL-4 in the (OVA + TMA)/(OVA + TMA) group in BALF. However, the inconsistent result is complex, it's not clear whether the result was affected by the experimental operation, or it possibly suggests that additional mechanisms play a role in this process. The cause was worthy of further research in the future.

The neural mechanisms involved in asthma have received much attention in recent years [37]. It was verified that TRPA1 is a key neuronal mediator of allergic airway inflammation [38]. By activating TRPA1, chemical irritants might trigger the release of neuropeptides and chemokines in the airways, thereby exacerbating the cellular and tissue inflammatory response observed. It was demonstrated that inhibition or genetic block of TRPA1, V1 channels did not result in the development of AHR in response to toluene-2,4-diisocyanate (TDI) challenge, and the levels of IgE, IL-13 were not increased even though the mice had been successfully sensitized [39]. Our previous research also found out that PM2.5 could exacerbate asthma mice sensitized with OVA through TRPs channels [19]. The results in this investigation showed that the gene and protein expressions of TRPA1, V1 and V2 significantly increased in the asthmatic mice treated by OVA plus TMA compared with the mice treated by single OVA or TMA. SP, as a sensory neuropeptide, is an essential mediator of airway inflammatory response [17]. Once the TRP channel opens, Ca^{2+} enters the cells, causing the release of tachykinins such as SP [40]. PGD_2 has many effects in the airways that may contribute to asthma pathophysiology including increased mucus production, vasodilation and capillary permeability [41,42]. NGF is secreted by a variety of nerve cells and immune cells. It can promote the release of inflammatory cells, modulates immune cells, and lead to airway inflammation, airway hyperresponsiveness and bronchial remodeling [43]. The results in this study had also shown that with the increase of gene and protein expressions of TRPA1, V1, V2, levels of IL-13, SP, PGD_2 and NGF also increased in the asthma model induced by OVA plus TMA. It suggested that TRPs and the cytokines, neurokines were involved in process of TMA aggravating asthma.

It was found that TMA aggravated OVA-induced asthma, which was indicated by the elevated levels of inflammation and related Th2 cytokines. Simultaneously, the increased gene and protein expression of

TRPA1, V1 V2 and the increased levels of SP, PGD_2, NGF were detected. The interesting findings were that TMA exacerbated asthma probably through regulating TRP channels, neurokines and Th2 cytokines. However, how the neurons, neurokines impact inflammation and immunology are less clear up to now. The mechanisms of neural and immune interaction under the regulation of TRP channels during aggravated asthma will be deeply explored in the future.

In conclusion, OVA plus TMA increased airway inflammatory, airway hyperresponsiveness and lung injury. The gene and protein expressions of TRPA1, V1, V2 were increased, with the higher level of IL-4 in supernatant of lung homogenate, higher levels of the IL-13, SP, PGD_2 and NGF in BALF and supernatant of lung homogenate were obtained. Through our previous and present study, it was found that environmental pollutants such as TMA, would aggravate asthma, and this exacerbation process might be related to the TRP channels. This study may provide the evidence that TRP channels could be the new targets and the use of TRP channel inhibitors would become a new approach for the clinical treatment of TMA aggravating asthma. At last, public health problems caused by environmental pollutants such as TMA exacerbating asthma should be paid more attention.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

Acknowledgments

This work was supported by National Natural Science Foundation of China (Grant No. 81473580, 81673864), the Priority Academic Program Development of Jiangsu Higher Education Institution (PAPD), Program of the Jiangsu Collaborative Innovation Center of CMRI and Jiangsu Key Laboratory for High Technology Research of Traditional Chinese Medicine Formulae.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.01.038.

References

[1] K.H. Kim, S.A. Jahan, E. Kabir, A review on human health perspective of air pollution with respect to allergies and asthma, Environ. Int. 59 (2013) 41–52.
[2] F. Liu, Y. Zhao, Y.Q. Liu, Y. Liu, P. Sun, M.M. Huang, Y. Liu, G.H. Dong, Asthma and asthma related symptoms in 23,326 Chinese children in relation to indoor and outdoor environmental factors: the seven northeastern cities (SNEC) study, Sci. Total Environ. 497 (2014) 10–17.
[3] B.N. Lambrecht, H. Hammad, The immunology of asthma, Nat. Immunol. 16 (1) (2015) 45–56.
[4] W.W. Busse, S. Holgate, E. Kerwin, Y. Chon, J. Feng, J. Lin, S.L. Lin, Randomized, double-blind, placebo-controlled study of brodalumab, a human anti–IL-17 receptor monoclonal antibody, in moderate to severe asthma, Am. J. Respir. Crit. Care Med. 188 (2013) 1294–1302.
[5] J. Tan, J.A. Bernstein, Occupational asthma: an overview, Curr Allergy Asthma Rep 14 (5) (2014) 431.
[6] S.J. Stocks, R. McNamee, H.F. van der Molen, C. Paris, P. Urban, G. Campo, R. Sauni, B.M. Jarreta, M. Valenty, L. Godderis, D. Miedinger, P. Jacquetin, H.M. Gravseth, V. Bonneterre, M. Telle-Lamberton, L. Bensefa-Colas, S. Faye, G. Mylle, A. Wannag, Y. Samant, T. Pal, S. Scholz-Odermatt, A. Papale, M. Schouteden, C. Colosio, S. Mattioli, R. Agius, I.S. Working Grp 2 Cost Action, Trends in incidence of occupational asthma, contact dermatitis, noise-induced hearing loss, carpal tunnel syndrome and upper limb musculoskeletal disorders in European countries from 2000 to 2012, Occup. Environ. Med. 72 (4) (2015) 294–303.
[7] M.S. Dykewicz, Occupational asthma: current concepts in pathogenesis, diagnosis, and management, J. Allergy Clin. Immunol. 123 (3) (2009) 519–528.
[8] L.C. Grammer, A.M. Ditto, A. Tripathi, K.E. Harris, Prevalence and onset of rhinitis and conjunctivitis in subjects with occupational asthma caused by trimellitic anhydride (TMA), J. Occup. Environ. Med. 44 (12) (2002) 1179–1181.
[9] R. Nishino, T. Fukuyama, Y. Watanabe, Y. Kurosawa, T. Koasaka, T. Harada, Detection of respiratory allergies caused by environmental chemical allergen via measures of hyper-activation and degranulation of mast cells in lungs of NC/Nga mice, J. Immunotoxicol. 13 (5) (2016) 676–685.
[10] T. Fukuyama, Y. Tajima, H. Ueda, K. Hayashi, Y. Shutoh, T.R. Saito, T. Harada, T. Kosaka, Investigation of the chemical-induced selective type II (TH2) allergic response in mice: effect of the length of the sensitizing phase, J. Immunotoxicol. 6 (2) (2009) 75–83.
[11] J.F. Regal, M.E. Mohrman, D.M. Sailstad, Trimellitic anhydride-induced eosinophilia in a mouse model of occupational asthma, Toxicol. Appl. Pharmacol. 175 (3) (2001) 234–242.
[12] B.F. Bessac, S.-E. Jordt, Sensory detection and responses to toxic gases: mechanisms, health effects, and countermeasures, Proc. Am. Thorac. Soc. 7 (4) (2010) 269–277.
[13] D.E. Clapham, TRP channels as cellular sensors, Nature 426 (6966) (2003) 517.
[14] B.F. Bessac, S.E. Jordt, Breathtaking TRP channels: TRPA1 and TRPV1 in airway chemosensation and reflex NS/NS, Physiology 23 (6) (2008) 360–370.
[15] D.E. Naumov, O.O. Kotova, A.G. Prikhodko, J.M. Perelman, V.P. Kolosov, Trpv2 gene polymorphisms are associated with hypoosmotic airway hyperresponsiveness in asthma, B34. Genetics and Genomics of Lung Disease, American Thoracic Society, 2017(pp. A3234-A3234).
[16] K. Matsumoto, T. Hosoya, K. Tashima, T. Namiki, T. Murayama, S. Horie, Distribution of transient receptor potential vanilloid 1 channel-expressing nerve fibers in mouse rectal and colonic enteric nervous system: relationship to peptidergic and nitrergic neurons, Neuroscience 172 (2011) 518–534.
[17] L. Cruz-Orengo, A. Dhaka, R.J. Heuermann, T.J. Young, M.C. Montana, E.J. Cavanaugh, D. Kim, G.M. Story, Cutaneous nociception evoked by 15-delta PGJ2 via activation of ion channel TRPA1, Mol. Pain 4 (1) (2008) 30.
[18] A.Z. El-Hashim, S.M. Jaffal, Nerve growth factor enhances cough and airway obstruction via TrkA receptor-and TRPV1-dependent mechanisms, Thorax 64 (9) (2009) 791–797.
[19] H. Liu, X. Fan, N. Wang, Y. Zhang, J. Yu, Exacerbating effects of $PM_{2.5}$ in OVA-sensitized and challenged mice and the expression of TRPA1 and TRPV1 proteins in lungs, J. Asthma 54 (8) (2017) 807–817.
[20] L. Xu, L. Shi, L. Feng, J. Yu, X. Fan, Study on the of occupational asthma model rats induced by trimellitic anhydride, Chin. J. Asthmology 6 (6) (2012) 410–414.
[21] A. Wallrapp, S.J. Riesenfeld, P.R. Burkett, R.-E.E. Abdulnour, J. Nyman, D. Dionne, M. Hofree, M.S. Cuoco, C. Rodman, D. Farouq, The neuropeptide NMU amplifies ILC2-driven allergic lung inflammation, Nature 549 (7672) (2017) 351.
[22] K. Oeser, J. Maxeiner, C. Symowski, M. Stassen, D. Voehringer, T cells are the critical source of IL-4/IL-13 in a mouse model of allergic asthma, Allergy 70 (11) (2015) 1440–1449.
[23] T. Chen, L. Xiao, L. Zhu, S. Ma, T. Yan, H. Ji, Anti-asthmatic effects of ginsenoside Rb1 in a mouse model of allergic asthma through relegating Th1/Th2, Inflammation 38 (5) (2015) 1814–1822.
[24] M.E. Munroe, T.R. Businga, J.N. Kline, G.A. Bishop, Anti-inflammatory effects of the neurotransmitter agonist honokiol in a mouse model of allergic asthma, J. Immunol. 185 (9) (2010) 5586–5597.
[25] G.D. Prete, Human Th1 and Th2 lymphocytes: their role in the pathophysiology of atopy, Allergy 47 (5) (1992) 450–455.
[26] B.N. Lambrecht, H. Hammad, The immunology of asthma, Nat. Immunol. 16 (1) (2015) 45.
[27] J.V. Fahy, Type 2 inflammation in asthma—present in most, absent in many, Nat. Rev. Immunol. 15 (1) (2015) 57.
[28] W.E. Paul, J. Zhu, How are TH2-type immune responses initiated and amplified? Nat. Rev. Immunol. 10 (4) (2010) 225.
[29] J.A. Vanoirbeek, M. Tarkowski, H.M. Vanhooren, V. De Vooght, B. Nemery, P.H. Hoet, Validation of a mouse model of chemical-induced asthma using trimellitic anhydride, a respiratory sensitizer, and dinitrochlorobenzene, a dermal sensitizer, J. Allergy Clin. Immunol. 117 (5) (2006) 1090–1097.
[30] M. Wegmann, Th2 cells as targets for therapeutic intervention in allergic bronchial asthma, Expert. Rev. Mol. Diagn. 9 (1) (2009) 85–100.
[31] N. Gour, M. Wills-Karp, IL-4 and IL-13 signaling in allergic airway disease, Cytokine 75 (1) (2015) 68–78.
[32] B.N. Lambrecht, H. Hammad, The immunology of asthma, Nat. Immunol. 16 (1) (2015) 45–56.
[33] R.W. Bottema, I.M. Nolte, T.D. Howard, G.H. Koppelman, A.E. Dubois, G. De Meer, M. Kerkhof, E.R. Bleecker, D.A. Meyers, D.S. Postma, Interleukin 13 and interleukin 4 receptor-α polymorphisms in rhinitis and asthma, Int. Arch. Allergy Appl. Immunol. 153 (3) (2010) 259–267.
[34] H. Matsumoto, Y. Hirata, K. Otsuka, T. Iwata, A. Inazumi, A. Niimi, I. Ito, E. Ogawa, S. Muro, H. Sakai, Interleukin-13 enhanced Ca2+ oscillations in airway smooth muscle cells, Cytokine 57 (1) (2012) 19–24.
[35] M.-H. Oh, S.Y. Oh, J. Lu, H. Lou, A.C. Myers, Z. Zhu, T. Zheng, TRPA1-dependent pruritus in IL-13–induced chronic atopic dermatitis, J. Immunol. 201 (6) (2013) 1300300.
[36] S.P. Hogan, A. Koskinen, K.I. Matthaei, I.G. Young, P.S. Foster, Interleukin-5–producing CD4+ T cells play a pivotal role in aeroallergen-induced eosinophilia, bronchial hyperreactivity, and lung damage in mice, Am. J. Respir. Crit. Care Med. 157 (1) (1998) 210–218.
[37] A. Konstantinidis, S. Barton, I. Sayers, I. Yang, J. Lordan, S. Rorke, J. Clough, S. Holgate, J. Holloway, Genetic association studies of interleukin-13 receptor α1 subunit gene polymorphisms in asthma and atopy, Eur. Respir. J. 30 (1) (2007) 40–47.
[38] A.I. Caceres, M. Brackmann, M.D. Elia, B.F. Bessac, D. del Camino, M. D'Amours, J.S. Witek, C.M. Fanger, J.A. Chong, N.J. Hayward, A sensory neuronal ion channel essential for airway inflammation and hyperreactivity in asthma, Proc. Natl. Acad. Sci. 106 (22) (2009) 9099–9104.
[39] F.C. Devos, B. Boonen, Y.A. Alpizar, T. Maes, V. Hox, S. Seys, L. Pollaris, A. Liston, B. Nemery, K. Talavera, Neuro-immune interactions in chemical-induced airway

hyperreactivity, Eur. Respir. J. 52 (3) (2016) (ERJ-01778-2015).
[40] L.M. Montaño, V. Carbajal, J.L. Arreola, C. Barajas-López, E. Flores-Soto, M.H. Vargas, Acetylcholine and tachykinins involvement in the caffeine-induced biphasic change in intracellular Ca2+ in bovine airway smooth muscle, Br. J. Pharmacol. 139 (6) (2003) 1203–1211.
[41] K. Alving, R. Matran, J. Lundberg, The possible role of prostaglandin D2 in the long-lasting airways vasodilatation induced by allergen in the sensitized pig, Acta Physiol. Scand. 143 (1) (1991) 93–103.
[42] Z. Marom, J.H. Shelhamer, M. Kaliner, Effects of arachidonic acid, mono-hydroxyeicosatetraenoic acid and prostaglandins on the release of mucous glyco-proteins from human airways in vitro, J. Clin. Invest. 67 (6) (1981) 1695–1702.
[43] V. Freund-Michel, N. Frossard, The nerve growth factor and its receptors in airway inflammatory diseases, Pharmacol. Ther. 117 (1) (2008) 52–76.