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ORIGINAL ARTICLE

Repeated exposure to temperature variation exacerbates airway inflammation through TRPA1 in a mouse model of asthma

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ABSTRACT

Background and objective: **Studies from epidemiology suggest that ambient temperature is one of the underlying triggers and potential causes of asthma. The aim of this study was to examine the impact and the molecular mechanism of temperature-invoked airway inflammation using an experimental model of asthma in BALB/c mice.**

Methods: **Mice were exposed to different temperature conditions (steady 26°C, 26°C/18°C cycle, 26°C/10°C cycle) and received sensitization and challenge of ovalbumin (OVA) during a 21-day period. HC030031, a selective transient receptor potential A1 (TRPA1) channel blocker, was used to investigate the underlying mechanism of TRPA1 in 'asthmatic' airways. After the final OVA challenge, in vivo lung function was measured, and bronchoalveolar lavage fluid (BALF) and pulmonary inflammation were assessed.**

Results: **The temperature variations, especially the largest temperature difference (16°C), exacerbated airway inflammation in OVA-induced mice, increasing the levels of serum total-IgE (immunoglobulin E) and IgG1, inflammatory cells and cytokines in BALF. Analysis of histopathological changes and lung function verified that repeated exposure to very cold and changed temperatures aggravated airway hyperresponsiveness (AHR). Significant upregulation of TRPA1 expression was revealed by immunohistochemistry in the presence of the largest temperature variation (26°C/10°C cycle), while administration of HC030031 successfully inhibited TRPA1 expression, thus attenuating the asthma-like pathological features.**

Conclusion: **Repeated exposure to temperature variation exacerbated experimental 'asthma' and TRPA1 mediated this temperature-dependent inflammatory effect.**

SUMMARY AT A GLANCE

We evaluated the effect of temperature variation on an ovalbumin (OVA)-induced mouse model of asthma and investigated the role of transient receptor potential A1 (TRPA1). The results showed that repeated exposure to temperature variation impaired lung function. TRPA1 upregulation exacerbated inflammatory responses, while TRPA1 inhibition presented as a potential therapeutic target.

Key words: airway inflammation, asthma, ovalbumin-induced mouse model, temperature variation, transient receptor potential A1.

INTRODUCTION

People in everyday life frequently experience temperature variations as a result of diurnal temperature changes or the use of indoor heating and cooling devices. Such temperature stimuli are believed to increase the incidence of temperature-related diseases[1,2] and increase respiratory symptoms among a sensitive subpopulation.[3,4] It is, therefore, of importance to understand the underlying mechanism to improve both individual and population health.

Asthma has dramatically increased in incidence over the last decade, currently affecting 235 million people worldwide.[5–7] According to epidemiological surveys, a cold environmental stimulus is believed a 'trigger' exacerbating existing asthma symptoms,[8] or may directly trigger asthma.[9] Hyrkäs *et al.*[10] surveyed 1623 young subjects and found that existing asthma increased the risks of all cold-weather related respiratory symptoms. Guo *et al.*[11] examined the association between cold spells and paediatric outpatient visits for asthma, where cold temperatures significantly increased asthma attacks among children. In addition, cross-sectional studies in Korea,[12] Australia[13] and Hong Kong[14] also confirmed an adverse association between a large diurnal temperature variation and the risk of

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asthma symptoms. A 1 °C change in temperature was associated with a 1% increase in asthma-related emergency department visits,[15] confirming the negative impact of temperature variation on asthma.

Cold air, coupled with temperature variations, has been shown to induce contraction of tracheal smooth muscle, decrease pulmonary circulation and lung perfusion and result in airway hyperreactivity,[16] while the mechanism remains incompletely understood. McKemy[17] argued that the transient receptor potential A1 (TRPA1) might be involved in cold air-induced cough in asthma, because an agonist of TRPA1 provoked coughing in healthy volunteers,[18] and it was more significant under inflammatory conditions.[19] The TRPA1 agonist, cinnamaldehyde, activates capsaicin-sensitive vagal neurons, causing pulmonary C-fibre discharge, and initiating respiratory irritation reflexes in mice.[20,21] Caceres *et al.*[22] found that airway inflammation and airway hyperresponsiveness (AHR) were reduced in TRPA1$^{-/-}$ asthmatic mice, while treatment with the TRPA1 antagonist HC-030031 significantly reduced cytokines in the airway epithelium.[22] Consequently, it is inferred that TRPA1 may sense temperature and mediate airway inflammatory responses. However, whether and how TRPA1 responds to temperature stimulus in asthma in vivo remains unclear.

To the authors' knowledge, no comprehensive studies have addressed the relationship between temperature variation and asthma, nor the potential molecular mechanism underlying the TRPA1 channel. As an ovalbumin (OVA)-induced mouse model is well recognized as a model of allergic asthma,[23] this study, based on that model, explores whether temperature variations promote airway inflammation. Further, through introducing a TRPA1 blocker, we examined whether the TRPA1 senses temperature stimuli and mediates inflammatory pathways, aiming to identify the potential role of TRPA1 channel in temperature-dependent asthma.

METHODS

Animal model

Specific Pathogen Free (SPF) male BALB/c mice (5–6 weeks) were purchased from Hubei Experimental Animal Center (Wuhan, China). Mice were housed in an environment-controlled room (25 ± 0.5°C and 50–70% relative humidity) for 1 week to acclimatize. All experimental protocols were approved by the Office of Scientific Research Management of Central China Normal University (approval ID: CCNU-IACUC-2016-003).

Experimental design

The experiment was designed to simulate the temperature variations to which people are exposed daily in winter when indoor heating is available. A total of 48 mice were allocated into six groups (eight mice/group) according to designs and treatments: (I) Saline-26°C group; (II) OVA-26°C group; (III) OVA-26°C/18°C group; (IV) OVA-26°C/10°C group; (V) OVA+HC030031-26°C group; and (VI) OVA+HC030031-26°C/10°C group (Fig. S1, Supplementary Information).

To enable OVA sensitization and challenge and TRPA1 blocking, a 21-day experimental period was designed. On days 1, 4, 7, 10 and 13, mice in groups II–VI were sensitized with OVA (50 μg OVA with 1.75 mg aluminium hydroxide as an adjuvant in a total volume of 300 μL of saline). From day 14 to day 21, all mice in groups II–VI were challenged with an aerosol of 1% OVA (OVA: saline, 1:99 w/v) for 30 min using an ultrasonic nebulizer (Yuyue 402A Type I, Jiangsu Yuyue Medical Equipment & Supply Co., Ltd., Nanjing, China). In addition, mice in groups V and VI were given HC-030031 intraperitoneally (30 mM HC-030031 stock in DMSO and 0.2% Tween 80 in saline) at a dose of 30 mg/kg/per every 5 days from day 1 to day 16.

All mice were fed in the climate chamber (BD-PRX-150A, Hangzhou Jtone Electronic Co.Ltd., Hangzhou, China). For groups I, II and V, the temperature was set at 26°C. For group III, it was set at 26°C for 12 h and 18°C for 12 h. For groups IV and VI, the temperature was set at 26°C for 12 h and 10°C for 12 h. During the experiments, the mice had access to water and food ad libitum.

Serum Ig and BALF cytokines

Mice were sacrificed 24 h after the final OVA challenge. Blood samples were obtained by heart puncture. The blood was kept at room temperature for 30 min and then centrifuged (24°C, 3000 rpm, 15 min). Enzyme-linked immunosorbent assay (ELISA) kits for total-IgE (immunoglobulin E; limit of detection 4 ng/mL) and the OVA-specific IgG1 (0.1 mg/mL) were obtained from BlueGene (Shanghai, China). Concentrations were determined in duplicate for each sample.

Ice-cold saline of 1 mL was instilled into the trachea and lung three times, and the entire process was repeated twice to obtain approximately 1.5 mL bronchoalveolar lavage fluid (BALF). Supernatants of the BALF samples were collected after centrifugation (1000 rpm, 4°C, 10 min) and stored (−80°C). The cell pellets were later re-suspended using 500 μL saline, and inflammatory cells were counted using a haematology analyser (MTN-21, China).

BALF supernatants were thawed to room temperature before use. Cytokine levels were determined using commercial ELISA kits from eBioscience (San Diego, CA, USA) according to the manufacturer's instructions. The lowest detection limits for IL-4, IL-6, IL-13 and IL-17 were 4 pg/mL, 15 pg/mL for interferon-gamma (IFN-γ) and 1.0 pg./mL for granulocyte-macrophage colony-stimulating factor(GM-CSF).

Histological analysis

Tissue from the lung biopsies was fixed in 4% formaldehyde and embedded in paraffin. Slices of 3-μm thick were mounted onto slides, and stained with haematoxylin and eosin(HE), Periodic Acid-Schiff (PAS) and Masson's Trichrome (MT). For immunohistochemistry, after dewaxing, rehydration and antigen retrieval, the sections were incubated with 0.3% hydrogen peroxide and blocked by appropriate normal serum. Sections were subsequently incubated overnight at 4°C with monoclonal antibodies: anti-Mast Cell Tryptase (1:100; Abcam, MA, USA) and anti-TRPA1 (1:100, Abcam). The reaction product was visualized by Diaminobenzidine

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(DAB) complexes. The negative control was obtained by omitting the primary antibody. Sections were washed again, counterstained with haematoxylin, dehydrated, cleared and mounted in DPX (Sigma-Aldrich Corp., St.Louis, MO, USA).

Airway hyperresponsiveness

An identical experimental protocol was repeated for another six groups (8 mice/group, totally 48 mice) to measure lung function using an AniRes 2005 lung function system (Beijing Bestlab High-Tech Co., Ltd., Beijing, China). Mice were anaesthetized with pentobarbital sodium (CAS no. 57-33-0, Sigma-Aldrich) at a dose of 100 mg/kg by intraperitoneal injection. The respiratory rate was pre-set at 90 breaths/min and the ratio of expiration/inspiration at 1.5:1. Methacholine (MCH, O-acetyl-β-methylcholine chloride, Sigma-Aldrich) was injected into the jugular vein with doses of 0.025, 0.05, 0.1 and 0.2 mg/kg successively every 5 min. The inspiratory resistance (Ri), expiratory resistance (Re) and lung dynamic compliance (Cldyn) were recorded using the AniRes2005 software (Beijing Bestlab High-Tech Co., Ltd, Beijing, China).[24]

Statistical analysis

Data in each group were presented as mean ± SD of all mice. An independent sample t-test was used to determine the differences between the six groups. Analysis of variance (ANOVA) with post hoc analysis was employed to test the differences by temperature variable. A *P*-value of <0.05 was considered statistically significant.

RESULTS

Changes in lung histopathology

Histopathological changes of mice are shown in Figure 1. Compared to Saline-26°C, there was a remarkable difference in the bronchial epithelium thickness, inward folding and inflammatory cell infiltration around the airways in the OVA-26°C group (HE staining; Fig. 1A-II), which were more obvious in the OVA-26°C/18°C and OVA-26°C/10°C groups (Fig. S2, Supplementary Information). In addition, the PAS-positive mucus cell staining (Fig. 1B, pale pink colour) and the subepithelial collagen deposition were increased (MT staining; Fig. 1C, blue colour) in the OVA-26°C/10°C group. After TRPA1 blocking, Figure 1A-VI shows less airway wall thickening and folding, and inflammatory cell infiltration in the OVA +HC030031-26°C/10°C group compared to Figure 1A-IV (HE staining in Fig. 1A). The degree of subepithelial collagen deposition under the lamina propria in Figure 1C-VI was less with MT staining compared to Figure 1C-IV in OVA-26°C/10°C group.

Serum Ig levels

The levels of total-IgE and IgG1 in serum are presented in Figure 2. Compared to Saline-26°C group, there were significant increases for the OVA-induced groups ($P < 0.05$). The levels of total-IgE and IgG1 were nearly fourfold and threefold higher, respectively, in the OVA-26°C group compared to Saline-26°C group, and even sevenfold and fourfold higher in the OVA-26°C/10°C group. By contrast, with the administration of HC030031, the levels of both total-IgE and IgG1 were significantly lower in the OVA+HC030031-26°C/10°C group in Figure 2, compared to the levels of total-IgE and IgG1 in OVA-26°C/10°C group ($P < 0.05$).

BALF inflammatory cell count and immunohistochemistry

Figure 3A, B shows the number of total BALF cells, lymphocytes (Lym), neutrophils (Neu), and eosinophils (Eos) in the six groups. The levels of total BALF cells, Lym, Neu and Eos increased in OVA-26°C and OVA +HC030031-26°C groups compared to Saline-26°C group, but they were not statistically significant ($P > 0.05$). However, compared to control Saline-26°C group, significant differences in the levels of total BALF cells, Lym, Neu and Eos were found in the OVA-26°C/18°C and OVA-26°C/10°C groups ($P < 0.05$), suggesting that repeated exposure to temperature-varying environments with large differences significantly exacerbated asthmatic inflammation. The levels of these inflammatory cell were observed to be significantly reduced (total BALF cells by 25.9%, Lym by 30% and Eos by 29.7%) in the OVA+HC030031-26°C/10°C group, when taking the levels of total BALF cells, Lym, Neu and Eos in OVA-26°C/10°C group as a baseline. Figure 3C, D show the mast cell degranulation staining. There was no tan stain in the lung section of the Saline-26°C group, while the tan colours were deeper in the temperature-changed groups, particularly in the OVA-26°C/10°C group (Fig. 3C). However, the staining was lightened in the OVA+HC030031-26°C/10°C group. This was further verified by the average optical density assay of mast cell degranulation in Figure 3D.

TRPA1 expression assay

TRPA1 expression was examined by immunohistochemical staining (Fig. 4). TRPA1 upregulation was observed in the five experimental OVA groups compared to the controlled Saline-26°C (Fig. 4A), with significant differences in average optical density ($P < 0.05$) (Fig. 4B). However, with the pre-administration of HC030031, the degree of staining was ameliorated in the OVA+HC030031-26°C/10°C group compared to OVA-26°C/10°C group. This verified the activation and expression of TRPA1 caused by temperature variation stimulus while HC030031 successfully inhibited the TRPA1 expression.

BALF cytokines and pro-inflammatory factors

The levels of IL-4 and IFN-γ and other pro-inflammatory factors are shown in Table 1. Significant differences were found for IL-4, IFN-γ, IL-6, IL-13, IL-17 and GM-CSF in the OVA-induced groups compared to the Saline-26°C group ($P < 0.05$), and in the OVA-26°C/18°C and OVA-26°C/10°C groups compared to the OVA-26°C group ($P < 0.05$). This showed that repeated exposures to large temperature variations significantly increased the levels of inflammatory factors. However, compared with the levels of IL-4, IL-6, IL-13 and GM-CSF in the OVA-26°C/10°C group in Table 1, statistical analysis showed that in the OVA+HC030031-26°C/10°C group the levels

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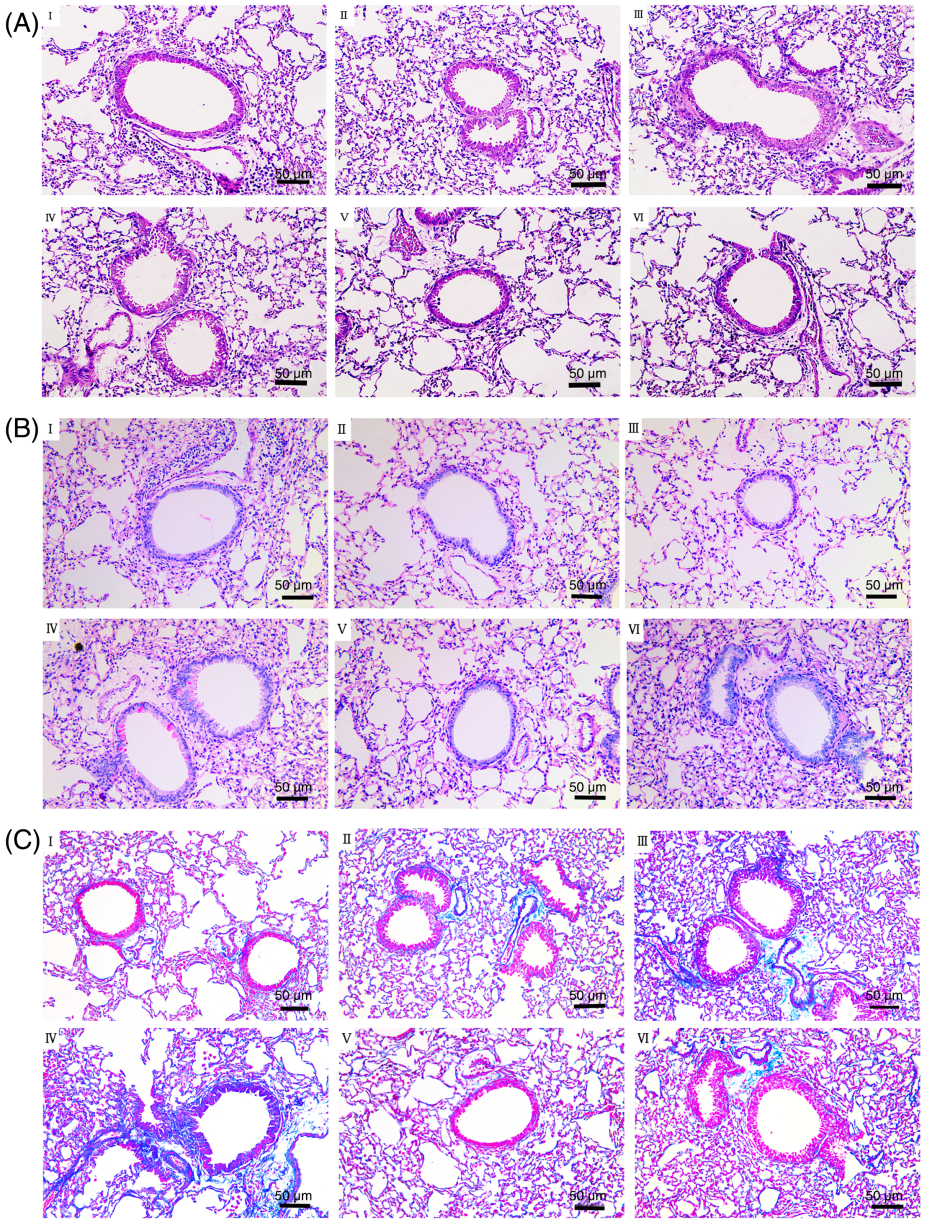


Figure 1 Histopathological analysis. (A) HE staining showing infiltration of inflammatory cells and hypertrophy of structural cells; (B) PAS staining showing the mucous-producing goblet cells (pale pink colour stain); and (C) MT staining showing subepithelial collagen deposition (blue colour stain). I: Saline-26°C; II: OVA-26°C; III: OVA-26°C/18°C; IV: OVA-26°C/10°C; V: OVA +HC030031-26°C; VI: OVA +HC030031-26°C/10°C (n = 8 per group). HE, haematoxylin and eosin; MT, Masson's Trichrome; OVA, ovalbumin; PAS, Periodic Acid-Schiff.

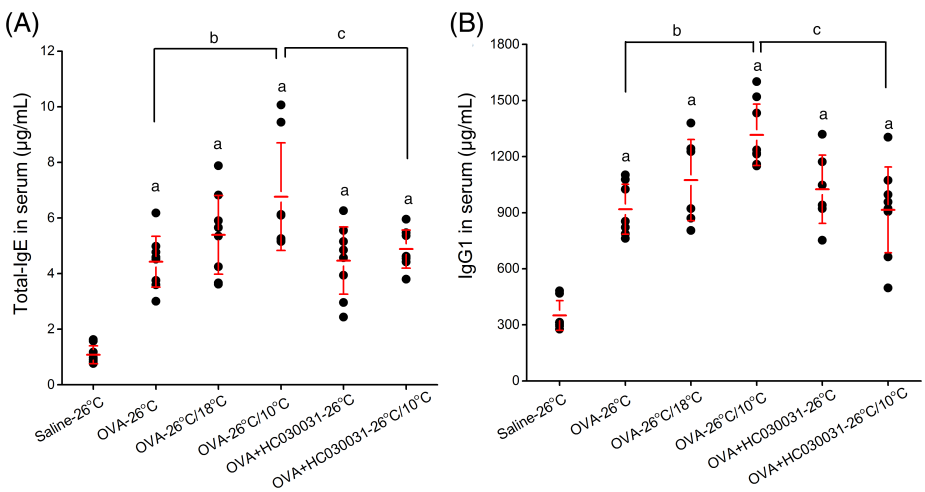


Figure 2 Serum concentrations of total-IgE and IgG1 in (A) total-IgE level and (B) IgG1 level. Data are expressed as mean ± SD (n = 8 per group). $^{a}P < 0.05$, compared with the Saline-26°C group; $^{b}P < 0.05$, compared with the OVA-26°C group; $^{c}P < 0.05$, comparison between the OVA-26°C/10°C and the OVA +HC030031-26°C/10°C. Ig, immunoglobulin; OVA, ovalbumin.

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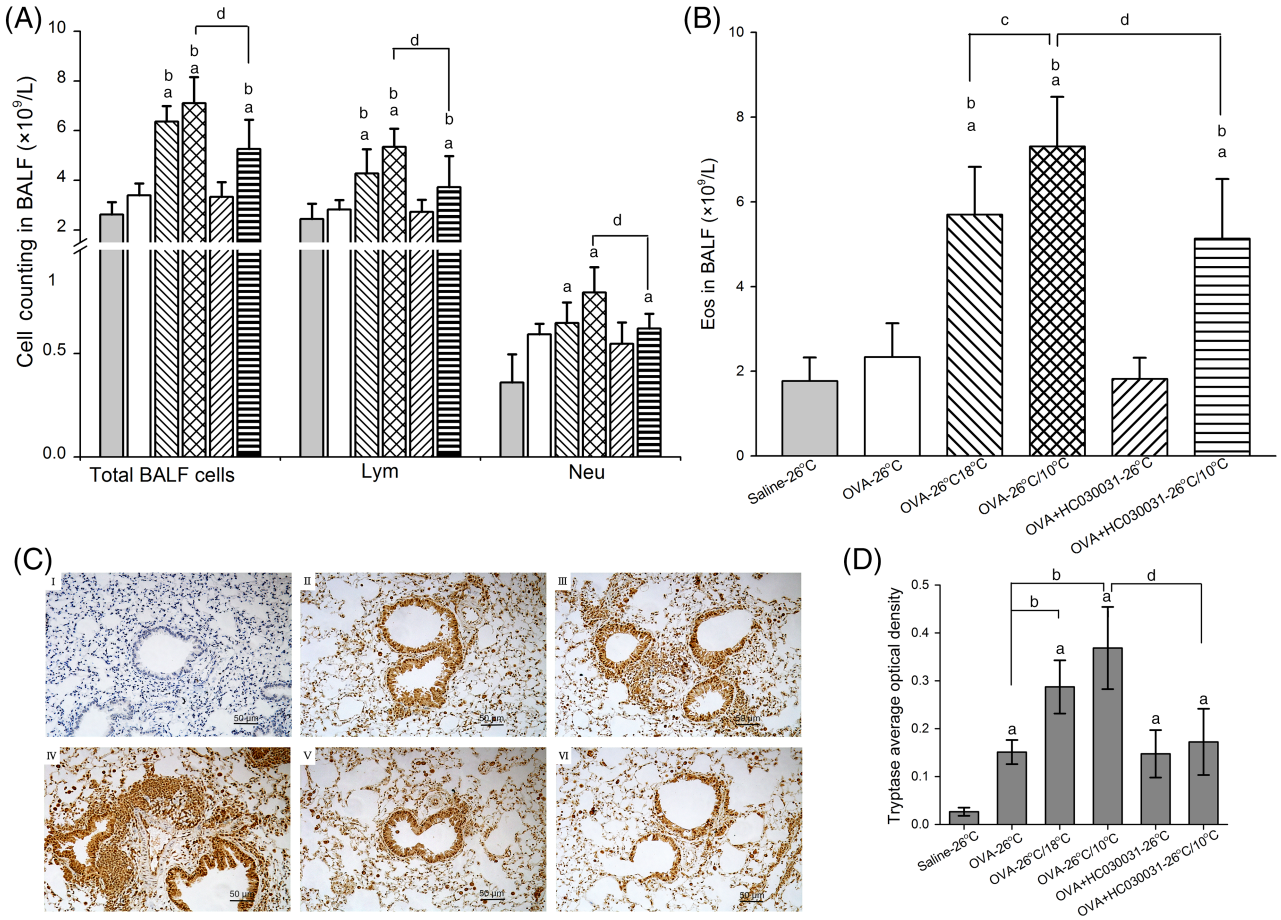


Figure 3 BALF inflammatory cell count and immunohistochemical analysis. (A) Cell count for total BALF cells, Lymphocytes (Lym) and Neutrophils (Neu). (B) Cell count for Eosinophils (Eos). (C) Immunohistochemistry for mast cell degranulation (tan colour staining). I: Saline-26°C (▬); II: OVA-26°C (▭); III: OVA-26°C/18°C (▨); IV: OVA-26°C/10°C (▩); V: OVA+HC030031-26°C (▱); VI: OVA +HC030031-26°C/10°C (▤). (D) The average optical density assay. Data are expressed as mean ± SD (n = 8 per group). [a]$P < 0.05$, compared with the Saline-26°C group; [b]$P < 0.05$, compared with the OVA-26°C group; [c]$P < 0.05$, comparison between the OVA-26°C/18°C and the OVA-26°C/10°C groups; [d]$P < 0.05$, comparison between the OVA-26°C/10°C and the OVA+HC030031-26°C/10°C groups. BALF, bronchoalveolar lavage fluid; OVA, ovalbumin.

of IL-4, IL-6, IL-13 and GM-CSF were significantly inhibited by the administration of HC030031 ($P < 0.05$), suggesting that the TRPA1 blocking attenuated the asthma-like pathology (Fig. S3, Supplementary Information).

Figure 5 shows the airway responses of mice. Both the Ri and Re increased with an increasing dose of MCH (Fig. 5A,B), and they were more significant at higher MCH doses. With an MCH dose of 0.1 and 0.2 mg/kg, the Ri and Re showed significant increases in the OVA-26°C/18°C and OVA-26°C/10°C groups ($P < 0.05$) while they decreased noticeably in the OVA +HC030031-26°C/10°C group ($P < 0.05$) compared to the Saline-26°C group. The pulmonary compliance was much lower when the temperature changed periodically (Fig. 5C), indicating that the temperature variations significantly affected the asthmatic airway structures.

DISCUSSION

Although inhalation of cold air is recognized as causing epithelial injury and mucosal inflammation, the regulatory mechanism in vivo is not adequately characterized. Our study shows that repeated exposures to varying temperatures increase the levels of IL-4 and IL-13, along with other pro-inflammatory factors (Table 1), which are consistent with Davis *et al.*'s study.[25] In that case, the high levels of IL-4 and IL-13 result in an increase in total-IgE level[26] and temperature variation further promotes the development (Fig. 2A). Meantime, the increasing levels of IL-17, IL-6 and GM-CSF further enhance the function of IL-4 and IL-13.[27] Moreover, the IgG1, associated with protecting against allergic reactions in mice,[28] was regulated by IL-4 and enhanced by the large temperature variation (Fig. 2B). The inflammatory responses are directly observed in Figure 3 with inflammatory cell infiltration and mast cell degranulation in the airways.[29] The respiratory resistances and airway remodelling are also exacerbated (Figs 1,5), due to thickening of the airway wall,[30] uncoupling of airway contraction as well as excessive contraction of airway smooth muscle.[31]

The TRPA1, activated by cold temperatures etc.,[32] has been identified in primary sensory afferent nerves and small diameter nociceptive neurons to detect

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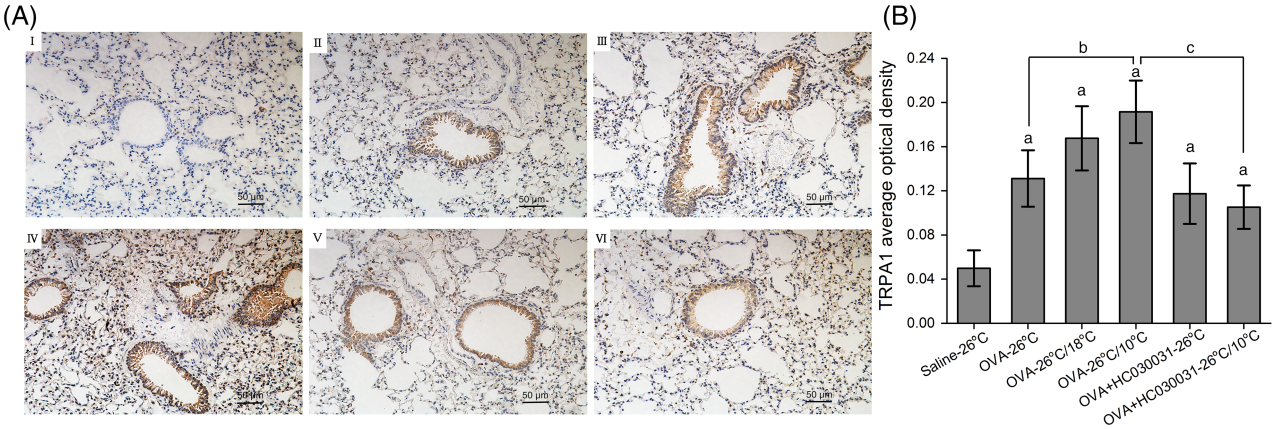


Figure 4 (A) Lung TRPA1 immunohistochemistry. I: Saline-26°C; II: OVA-26°C; III: OVA-26°C/18°C; IV: OVA-26°C/10°C; V: OVA +HC030031-26°C; VI: OVA+HC030031-26°C/10°C. (B) The average optical density assay. $^{a}P < 0.05$, compared with the Saline-26°C group; $^{b}P < 0.05$, compared with the OVA-26°C group; $^{c}P < 0.05$, comparison between the OVA-26°C/10°C and the OVA +HC030031-26°C/10°C groups. OVA, ovalbumin; TRPA1, transient receptor potential A1.

Table 1 Levels of cytokines and pro-inflammatory factors in BALF

Group	IL-4 (pg/mL)	IFN-γ (pg/mL)	IL-13 (pg/mL)	IL-6 (pg/mL)	IL-17 (pg/mL)	GM-CSF (pg/mL)
Saline-26°C	17.97 ± 3.57	37.78 ± 7.39	25.61 ± 6.0	15.71 ± 2.57	114.88 ± 10.8	30.22 ± 1.97
OVA-26°C	30.53 ± 8.22	36.84 ± 6.02	47.92 ± 15.1	24.17 ± 3.54	164.99 ± 21.15	49.27 ± 5.22
OVA-26°C/18°C	38.58 ± 6.46	22.38 ± 1.35	64.87 ± 6.22^{a}	29.15 ± 4.78	185.29 ± 33.76	56.16 ± 7.74^{a}
OVA-26°C/10°C	50.5 ± 4.62^{a}	33.74 ± 7.52^{a}	84.54 ± 9.28^{a}	35.69 ± 4.62^{a}	209.86 ± 31.72^{a}	72.19 ± 9.2^{a}
OVA + HC030031-26°C	34.88 ± 8.82	24.46 ± 6.69	41.16 ± 8.34	26.53 ± 3.76	179.99 ± 25.44	37.39 ± 3.25
OVA + HC030031-26°C/10°C	28.72 ± 5.45^{b}	28.0 ± 8.24^{b}	49.99 ± 7.16^{b}	24.56 ± 5.62^{b}	200.62 ± 25.78^{a}	49.93 ± 8.76^{b}

$^{a}P < 0.05$, compared with OVA-26°C group; $^{b}P < 0.05$, comparison between OVA-26°C/10°C and OVA+HC030031-26°C/10°C groups. Data are expressed as mean ± SD ($n = 8$).

BALF, bronchoalveolar lavage fluid; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN-γ, interferon-gamma; OVA, ovalbumin.

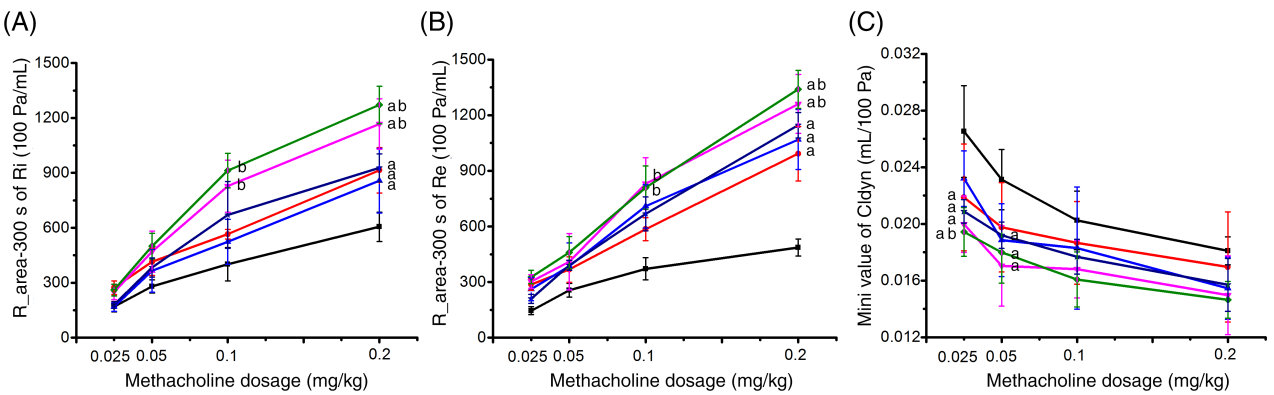


Figure 5 Airway hyperresponsiveness with different MCH dosages. (A) Ri, (B) Re and (C) Cldyn. Data are expressed as mean ± SD ($n = 6$–8 per group). $^{a}P < 0.05$, compared with the Saline-26°C group; $^{b}P < 0.05$, compared with the OVA-26°C group. —■—, Saline-26°C; —●—, OVA-26°C; —▼—, OVA-26°C/18°C; —◆—, OVA-26°C/10°C; —▲—, OVA+HC030031-26°C; —★—, OVA+HC030031-26°C/10°C. Cldyn, lung dynamic compliance; MCH, methacholine; OVA, ovalbumin; Re, expiratory resistance; Ri, inspiratory resistance.

noxious stimuli and mediate inflammatory hyperalgesia and neurogenic inflammation.[33] In this study, we focus primarily on the crucial role of TRPA1 to sense temperature stimuli in vivo and examine TRPA1 function through blocking. Our results demonstrated that when the temperature changed by a large amount (i.e. from 26°C to 10°C, to 26°C periodically), significant increase in inflammation was observed in OVA-challenged mice, while the increase was ameliorated by TRPA1 blockade. This suggests that the TRPA1 might sense

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large temperature variations and exacerbate temperature-induced allergic inflammation. TRPA1 blockade following OVA challenge and large environmental temperature fluctuation inhibits inflammatory signalling pathways, and thus attenuates the inflammatory responses, which accords with previous studies.[22,34] As the activation of TRPA1 would initiate inflammatory signalling pathways, resulting in a Ca^{2+} influx,[35] this effect is likely to be at the level of sensory nerves, due to HC030031 inhibiting the Bradykinin (BK)-induced sensory neuron, Ca^{2+} flux and vagus nerve depolarization.[36]

There is increasing evidence of two TRP channels, that is TRPA1 and Transient Receptor Potential Vanilloid 1 (TRPV1), mediating inflammatory responses.[37,38] They are distributed widely within the respiratory tract to increase the excitability of C-fibres during airway inflammation.[34] The bronchial C-fibres are thus activated by inflammatory mediators and release various neuropeptides, in particular Substance P (SP) and Calcitonin Gene-related Peptide (CGRP).[39] Wu *et al.*[40] experimentally revealed that TRPA1 and TRPV1 antagonists reduced the allergen-induced inflammation, pulmonary tissue damage and AHR, via suppression of neuropeptide release including SP and CGRP, which suggests a potential mechanism of temperature-induced airway inflammation in this study. However, as the TRPV1 was alternatively activated by elevated temperatures (>42°C),[41] one study in mice revealed that TRPV1 was a major mediator of inflammatory thermal hyperalgesia and inflammatory painful hypersensitivity to hot stimuli.[42] Given the current study focuses more on cold temperatures, the significantly negative effect on asthma with TRPA1 upregulation is verified in OVA-26°C/10°C group, which promotes the activation of T helper cell 2 (Th2) cells, the synthesis of cytokines and IgE and tissue remodelling and AHR (Figs 2–5). In this case, the administration of HC030031 in OVA+HC030031-26°C/10°C group inhibits the sensory nerve excitability and thus the upregulation of TRPA1 activity, ameliorating the allergic inflammation. In fact, as all major neuronal inflammatory signalling pathways converge on TRPV1 and TRPA1,[34] the co-expression of TRPA1 and TRPV1 is believed to have coupling roles mediating airway inflammation to cold and hot stimuli. Therefore, more studies are needed to identify further the TRPV1- and TRPA1-mediated mechanisms.

Limitations should be noted with respect to the interpretation of this study. The study currently verifies TRPA1 involvement in airway inflammation caused by temperature variations. The question of whether the increasing inflammation is a result of large temperature fluctuations or of the coldest temperatures remains to be explored. As TRPA1 is expressed in subsets of sensory neurons in the central and peripheral systems,[32] whether the TRPA1 channel is directly activated by cold or its activation is an indirect effect needs to be examined.

In conclusion, this study highlights the significant effect of repeated exposure to temperature variations on the aggravation of an existing experimental model of asthma in vivo. The upregulation of TRPA1 mediates inflammatory pathways and exacerbates pulmonary dysfunction, while TRPA1 inhibition attenuates airway injury and asthma-like features. As the TRP channels are highly conserved between animals and human,[43] these results are expected to provide an insight into the role of TRPA1 in temperature variation-invoked human asthma. Further studies are needed to better characterize TRPA1 in airway inflammation, which may be beneficial for the potential protection and treatment of asthmatic populations.

Acknowledgements

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Abbreviations: AHR, airway hyperresponsiveness; BALF, bronchoalveolar lavage fluid; CGRP, calcitonin gene-related peptide; Cldyn, lung dynamic compliance; Eos, eosinophils; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN-γ, interferon-gamma; IL, interleukin; Lym, lymphocytes; MCH, methacholine; MT, Masson's Trichrome; Neu, Neutrophils; OVA, ovalbumin; PAS, Periodic Acid-Schiff; Re, expiratory resistance; Ri, inspiratory resistance; SP, SP-substance P; TRPA1, transient receptor potential A1; TRPV1, transient receptor potential vanilloid 1.

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Supplementary Information

Additional supplementary information can be accessed via the *html* version of this article at the publisher's website.

Figure S1 The schematic diagram for treatments of six different experimental groups.
Figure S2 The histological sections of HE, PAS, MT and quantitative analysis.
Figure S3 The levels of typical cytokines and pro-inflammatory factors in BALF.
Visual Abstract Repeated exposure to temperature variation exacerbates airway inflammation through TRPA1.

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Repeated exposure to temperature variation exacerbates airway inflammation through TRPA1

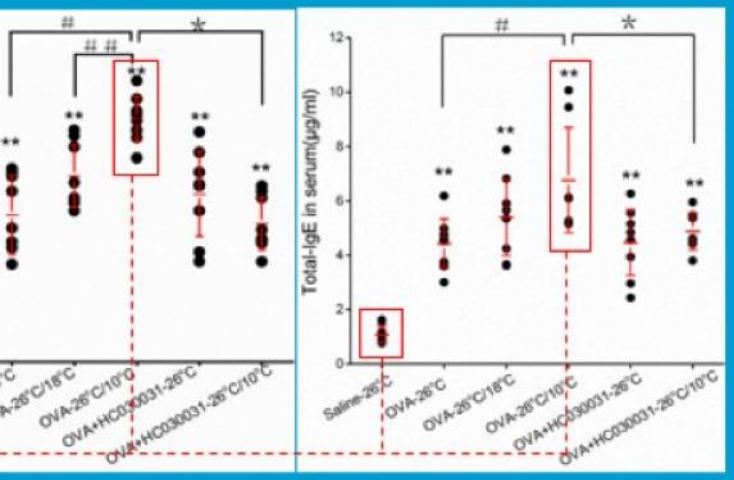
Different temperature conditions (1-20) : 26-26℃ / 26-18℃ / 26-10℃ | **Aerosolization: 1% OVA 15-21 (30 min)**

Day: 1&* 2 3 4* 5 6& 7* 8 9 10* 11& 12 13* 14 15 16& 17 18 19 20 21 22

Injected i.p. * : 0.02%OVA 300μl/per &: TRPA1 blocking HC030031 (30mg/kg/per)

Impact of TRPA1 mediated temperature-invoked airway inflammations in a mouse model of asthma:

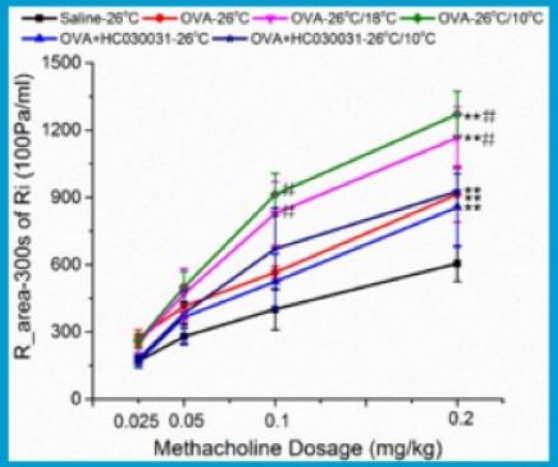
Inflammation in asthmatic airway



IL-4 17.97 → **50.50** (BALF(pg/ml))

IgE 1.08 → **6.76** (Serum(μg/ml))

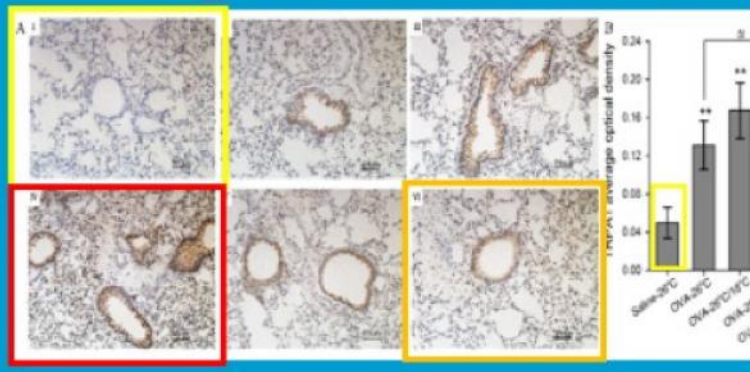
Airway hyper-responsiveness



Control → **Exposure: 2-fold**

(Inspiratory resistance measurement)

TRPA1 expression in lung



TRPA1 average optical density:

Control:

Temp variation 26 °C /10 °C:

Temp variation 26 °C /10 °C & TRPA1 blocking :

Respirology

Repeated exposure to temperature variation exacerbates airway inflammation through TRPA1 in a mouse asthma m

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SUPPLEMENTARY INFORMATION

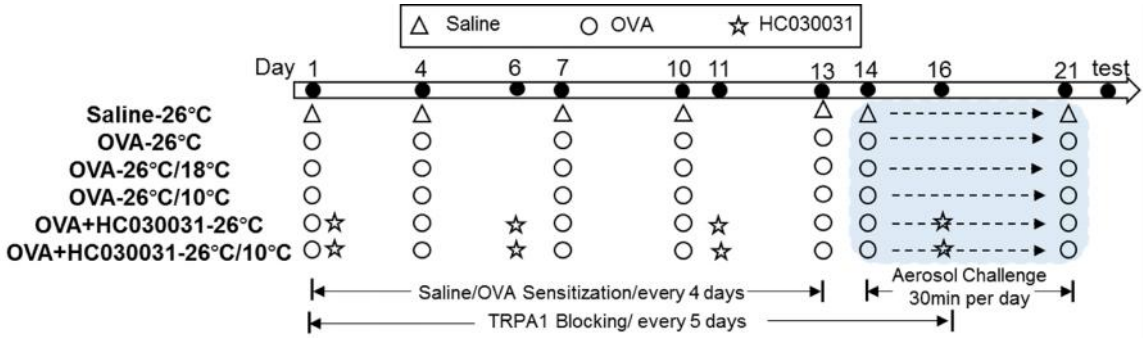
Repeated exposures to temperature variation exacerbate the airway inflammations through TRPA1 in a mouse model of asthma

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Figure S1- *The schematic diagram for treatments for six different experimental groups*



In everyday life, people frequently encounter large temperature variations resulting from outdoor diurnal temperature changes or the use of indoor heating and cooling devices. For instance, in the winter in severe cold zones, the outdoor temperature is always as low as −20◦C while the heating temperature is around 20◦C, resulting in a temperature gap up to 40◦C or larger. For majority, it is common that the indoor temperature is 26^{o}C while the outdoor temperature is 10^{o}C. However, little attention is paid to the effect of such winter case on human health, where the temperature gap can be large. Therefore, referring to the possible environmental exposures of people in daily life, the neutral (26^{o}C), mild cold (from 26^{o}C to 18^{o}C, to 26^{o}C) and moderate cold (from 26^{o}C to 10^{o}C, to 26^{o}C) exposures were considered. Based on the design, mice in 6 group are fed in the small climate chamber with the pre-set temperature conditions. The details are as follows:

(I)Saline-26 ^{o}C group, sensitization and challenge with saline, housed at 26 ^{o}C;

(II)OVA-26 ^{o}C group, sensitization and challenge with OVA, housed at 26 ^{o}C;

(III) OVA-26 ^{o}C/18 ^{o}C group, sensitization and challenge with OVA, housed at 26 ^{o}C/18 ^{o}C cycle;

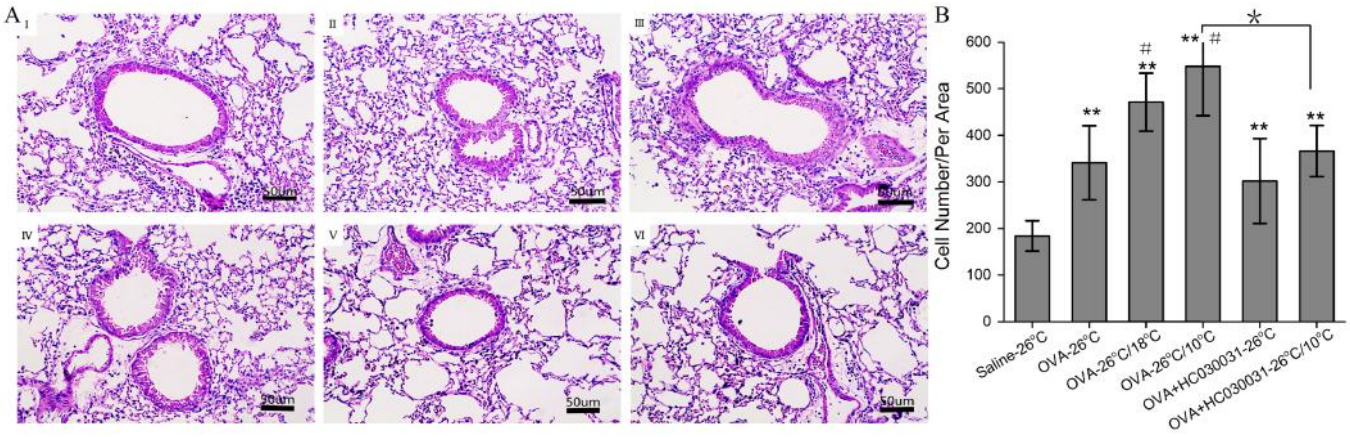
(IV) OVA-26 ^{o}C/10 ^{o}C group, sensitization and challenge with OVA, housed at 26 ^{o}C/10 ^{o}C cycle;

(V) OVA+HC030031-26 ^{o}C group, sensitization and challenge with OVA and TRPA1 blocker, housed at 26 ^{o}C;

(VI) OVA+HC030031-26 ^{o}C/10 ^{o}C group, sensitization and challenge with OVA and TRPA1 blocker, housed at 26 ^{o}C/10 ^{o}C cycle.

The period is 21 days, according to the different treatments of mice sensitization and challenge, as well as the TRPA1 blocking.

Figure S2-*The histological sections of H&E, PAS, MT and quantitative analysis*

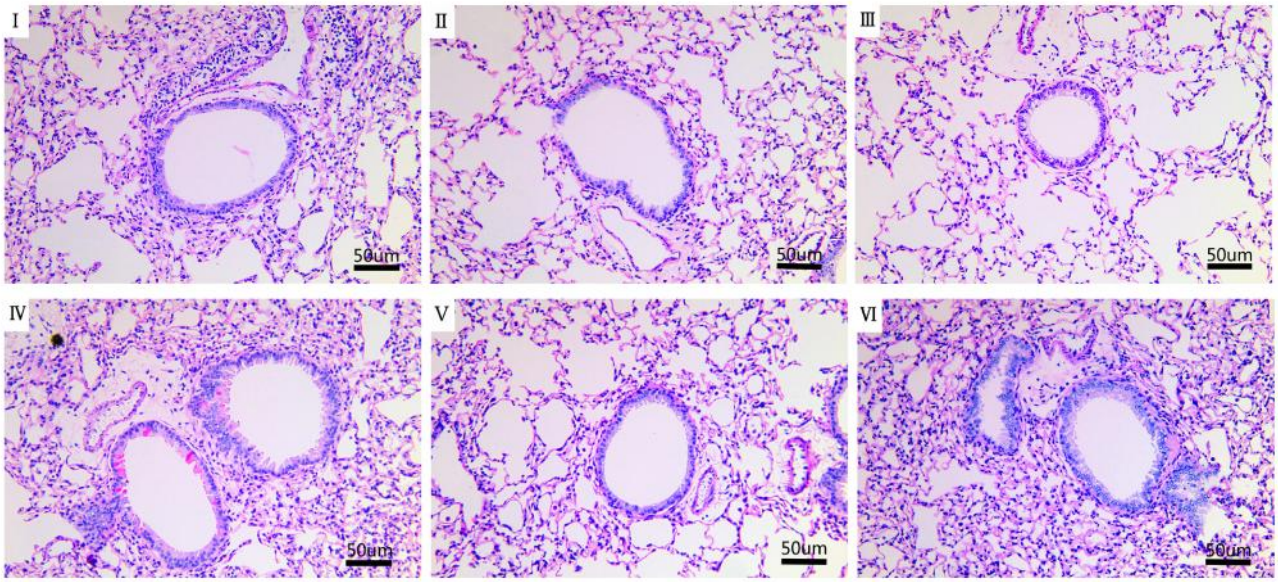


(I: Saline-26 ^{o}C; II: OVA-26 ^{o}C; III: OVA-26 ^{o}C/18 ^{o}C; IV: OVA-26 ^{o}C/10 ^{o}C; V: OVA+HC030031; VI: OVA+HC030031-26 ^{o}C/10 ^{o}C)

The tissues from the lung biopsies of mice were fixed in 4% formaldehyde and embedded in paraffin. Sections were cut at 3 µm, mounted onto slides, and stained with hematoxylin and eosin (H&E). Besides, the sections were viewed under a DM 4000B Microscope (1:50, Leica, Berlin, Germany) with camera and the cell number per area was counted. It shows that there is a significant difference in the bronchial epithelium thickness, increased inward folding and inflammatory cell infiltration around the airways in OVA-26^{o}C group compared to Saline-26^{o}C. The existence of thickening of the airway walls and folds, and inflammatory cell infiltration in the peribronchiolar regions are much more in evidence in Figures (A)-III and -IV, suggesting that the changes in ambient temperature significantly increase airway inflammation in asthmatic mice. However, the mice in group VI show comparatively less thickening of the airway wall and less folding, as well as less epithelium and inflammatory cell infiltration. This is quantified in Figure B that the cell count per area of H&E section increased significantly in OVA-induced groups and further enhanced in temperature-changed groups (see III: OVA-26 ^{o}C/18 ^{o}C; IV: OVA-26 ^{o}C/10 ^{o}C). Besides, there are significant differences of cell number per area between OVA-26 ^{o}C/10 ^{o}C and OVA+HC030031-26 ^{o}C/10 ^{o}C, suggesting the administration of HC030031 attenuates the infiltration of inflammatory cells.

The PSA, MT staining with large scale photo are presented on the next page.

PAS staining with pale pink color



MT staining with blue color

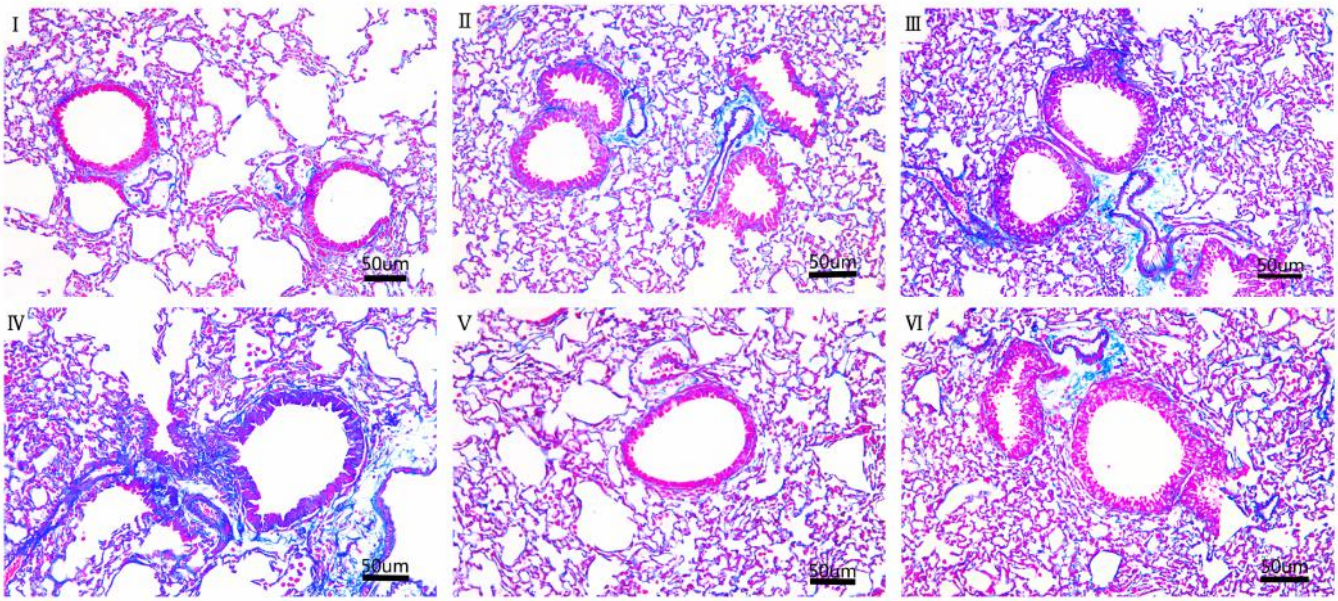
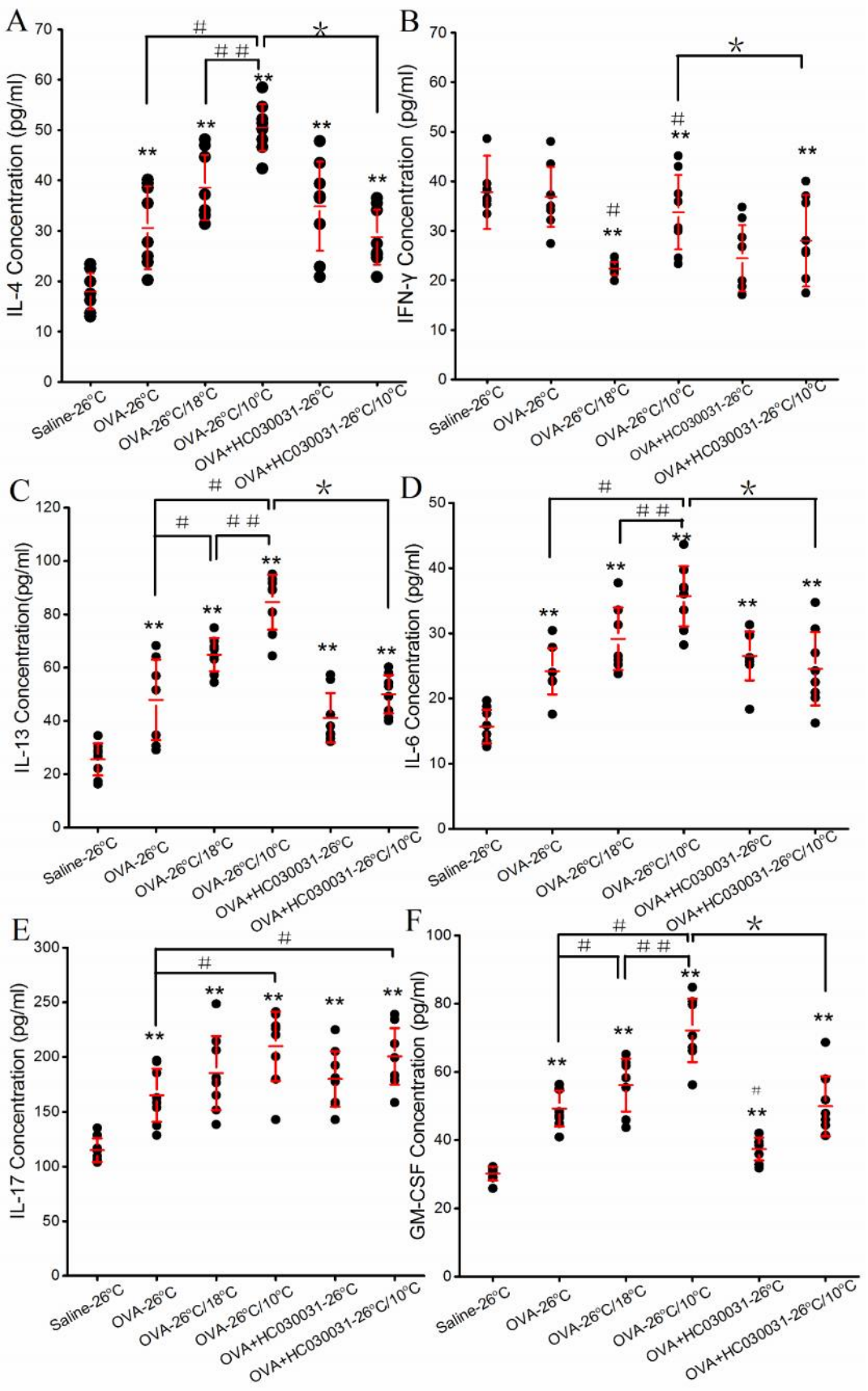


Figure S3- *The levels of typical cytokines and pro-inflammatory factors in BALF*



The bronchoalveolar lavage fluid (BALF) of all mice in each group were collected on day 21 and the typical cytokines were assayed using ELISA. A IL-4 level, B IFN-γ level, C IL-13 level, D IL-6 level, E IL-17 level, F GM-CSF level. Group data are presented as mean ±SD of 8 mice per group (n=8). **: $p<0.05$, compared with Saline-26 ^{o}C group; #: $p<0.05$, compared with OVA-26 ^{o}C group; ##: $p<0.05$, comparison between OVA-26 ^{o}C/18 ^{o}C and OVA-26 ^{o}C/10 ^{o}C; ∗ $p<0.05$, comparison between OVA-26 ^{o}C/10 ^{o}C and OVA+HC030031-26 ^{o}C/10 ^{o}C groups. All these inflammatory factors are higher when the temperature change with a large temperature difference, suggesting that temperature variation indeed promotes the inflammations in asthmatic airways. Besides, the HC030031 treatment in OVA+HC030031-26^{o}C/10^{o}C is observed that significant attenuates the levels of all inflammatory factors.