



Deficiency of voltage-gated proton channel Hv1 aggravates ovalbumin-induced allergic lung asthma in mice

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ABSTRACT

Asthma is a chronic airway inflammation that caused by many factors. The voltage-gated proton channel Hv1 has been proposed to extrude excessive protons produced by NADPH oxidase (NOX) from cytosol to maintain its activity during respiratory bursts. Here, we showed that loss of Hv1 aggravates ovalbumin (OVA)-induced allergic lung asthma in mice. The numbers of total cells, eosinophils and neutrophils in bronchoalveolar lavage fluid (BALF) of Hv1-deficiency (KO) mice are obviously increased after OVA challenge compared with that of wild-type (WT) mice. Histopathological staining reveals that Hv1-deficiency aggravates OVA-induced inflammatory cell infiltration and goblet cell hyperplasia in lung tissues. The expression of IL-4, IL-5 and IL-13 are markedly increased in lung tissues of OVA-challenged KO mice compared with that of WT mice. Furthermore, the expression levels of NOX2, NOX4 and DUOX1 are dramatically increased, while the expression levels of SOD2 and catalase are significantly reduced in lung tissues of OVA-challenged KO mice compared with that of WT mice. The production of ROS in lung tissues of KO mice is significantly higher than that of WT mice after OVA challenge. Our data suggest that Hv1-deficiency might aggravate the development of allergic asthma through increasing ROS production.

1. Introduction

Allergic asthma is a complex chronic airway inflammatory disease of lung caused by a variety of cells, especially mast cells, eosinophils, and T lymphocytes. The main characteristics of allergic asthma are airway hyperresponsiveness (AHR), infiltration of inflammatory cells into lung tissues, mucus hypersecretion by goblet cells and overexpression of Th2 cytokines including IL-4, IL-5, and IL-13 [1]. The main mechanism of allergic asthma is related to the imbalance of Th1/Th2 and Treg/Th17 cells ratios [2]. So far, allergic asthma is affecting more than 300 million people around the world, and this chronic respiratory disease seriously endangers human health. In the past 20 years, the morbidity and mortality caused by allergic asthma have been rising continuously, which has brought huge economic pressure to the public [3]. Therefore, it is urgent to find a way to treat and overcome asthma. Although the current understanding of the pathogenesis of asthma has made some progress, the effective treatment of refractory asthma inflammation is still limited.

Reactive oxygen species (ROS) play a double-edged role in physiological and pathophysiological processes. On the one hand, the low or medium concentration of ROS regulate the opening of ion channels, phosphorylation of proteins and activation of transcription factors [4]. On the other hand, the continuous high concentration of ROS may lead to non-specific damage of nucleic acid, lipid and protein, and then may cause the deterioration of many clinical diseases, including aging, cancer, inflammation, cardiovascular disease and neurodegeneration [5]. Besides mitochondria, the main source of intracellular ROS is NADPH oxidases (NOXs). The family of NOXs includes NOX1, NOX2, NOX3, NOX4, NOX5, DUOX1 and DUOX2 [6], which play important roles in regulating cellular processes, including cell differentiation, proliferation, growth, migration, contraction, apoptosis, and cytoskeleton regulation [7]. ROS, produced by NOXs, can directly or indirectly kill bacteria, fungi and microorganisms during host defense [8].

Hv1 is a voltage-gated proton channel, which has been proved to play an important role in innate immune cells such as phagocytes, and is

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related to the activity of NOXs and the production of ROS [9]. Recently, it has been shown that Hv1 is selectively expressed in lung epithelial cells and its function in the pulmonary epithelium is to reacidify the alkaline lung intimal fluid [10]. In the process of respiratory burst of phagocytes, Hv1 prevents depolarization of plasma membrane and excess acidification of cytosol by squeezing protons, to maintain the stability of intracellular pH [11–13]. However, the anti-inflammatory effect of Hv1 in asthma is not clear. Therefore, the potential roles and mechanisms of Hv1 in inflammation still need to be illustrated.

In the present study, we used Hv1-deficiency mice to investigate the anti-inflammatory effects and mechanisms of Hv1 in allergic lung asthma. We found that Hv1-deficiency effectively aggravated ovalbumin (OVA)-induced inflammatory cell recruitment, increased Th2 cytokine levels and goblet cells proliferation. Moreover, Hv1-deficiency dramatically increased OVA-induced ROS production in lung tissues and significantly increased the expression levels of NOXs while decreased the expression levels of anti-oxidases in lung tissues. Thus, we suggest that Hv1-deficiency aggravates OVA-induced allergic lung asthma in mice through increasing NOX-mediated ROS production.

2. Materials and methods

2.1. Chemicals and reagents

Ovalbumin (OVA; grade V) was purchased from Sigma. Mouse IL-4 and IL-5 enzyme-linked immunosorbent assay (ELISA) kits were obtained from Biolegend, and mouse IL-13 ELISA kit was obtained from eBioscience. N-acetylcysteine (NAC), mouse IL-10, IL-17A and IFN γ ELISA kits were obtained from Biolab. Imject® Alum, Periodic Acid Schiff (PAS) and Kwik-Diff staining kits were obtained from Thermo Fisher Scientific Inc. All other chemicals were of analytical-reagent grade.

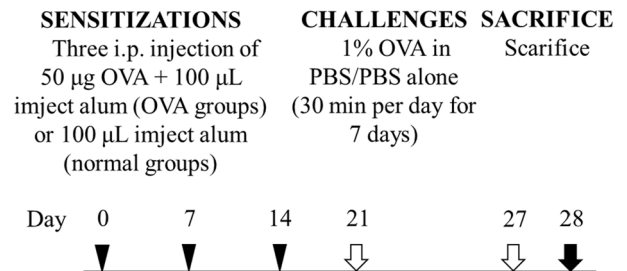
2.2. Animals

Mice bearing a targeted disruption in the Hv1 (KO mice, Hv1^{-/-}, backcrossed eight times) were kindly provided by Dr. Y. Okamura (School of Medicine, Osaka University), as previously described [14]. WT mice (Hv1^{+/+}) were of the same genetic background (C57BL/6J). All animals were housed in a controlled environment (24 $\pm$ 1 °C, 40–80% humidity) under specific pathogen-free conditions. Natural dark and light cycles (12 h) were maintained in animal house, along with standard feed and water ad libitum (Lillico Biotechnology). Experiments were performed with female mice, 6–8 weeks old, 18 $\pm$ 2 g, unless indicated otherwise. Before experimentation, mice were housed for a minimum of one week to adapt them to the environment. All animal husbandry and experiments were approved by and performed in accordance with guidelines from the Animal Research Committee of Nankai University.

2.3. Sensitization and challenge

Female C57BL/6J mice were randomly divided into the following groups: normal groups (WT control = WTC, KO control = KOC, n = 15), OVA groups (WT OVA control = WTO, KO OVA control = KOO, n = 15), the protocol was shown in Fig. 1A. Mice were sensitized by intraperitoneal (i.p.) injections of 50 μ g OVA in 100 μ L phosphate buffer saline (PBS) and then mixed with 100 μ L Imject® Alum to OVA groups or 100 μ L PBS mixed with 100 μ L Imject® Alum to normal groups on days 0, 7, and 14. Then from day 21, mice were challenged with 1% OVA in PBS (OVA groups) or PBS (normal groups) 30 min a day for 7 days consecutively, with a compressor nebulizer (flow rate was 6.7 L/min, ODARI, China).

A



B

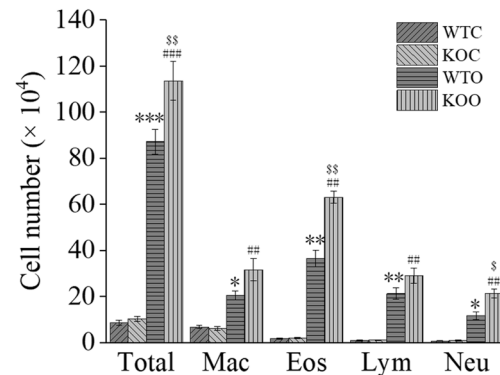


Fig. 1. Experimental protocol for allergic asthma models and effect of Hv1-deficiency on recruitment of inflammation cells in BALF and ROS production in lung tissues. (A) The mice were divided into four groups (n = 15 in each group) and sensitized via intraperitoneal injection of 50 μ g OVA in 100 μ L PBS and mixed with 100 μ L Imject® Alum on days 0, 7 and 14. Mice were challenged by 1% OVA in PBS for 30 min for 7 days. Mice were sacrificed 24 h after the last challenge. (B) BALF were collected 24 h after the last challenge and cells were counted. Eos = Eosinophils, Mac = Macrophages, Neu = Neutrophils, Lym = Lymphocytes. WTC = WT control, WTO = WT OVA, KOC = KO control, KOO = KO OVA. Values are shown as mean $\pm$ SEM (n = 6 for each group). * P < 0.05, ** P < 0.01, *** P < 0.001 vs. WTC; ## P < 0.01, ### P < 0.001 vs. KOC; \$ P < 0.05, \$\$ P < 0.01 vs. WTC.

2.4. Bronchoalveolar lavage fluid (BALF) collection

Twenty-four hours after the last inhalational exposure, the mice were anesthetized using pentobarbital sodium (50 mg/kg, i.p.), then the tracheas were cannulated and the BALF was collected and immediately centrifuged (800 rpm, 5 min at 4 °C). The supernatants were stored at -80 °C until used for cytokines assay, and the cell pellets were resuspended in PBS for total and differential cell counts. Total cell counts were counted on a hemocytometer. The number of eosinophils, neutrophils, macrophages and lymphocytes in BALF were counted on cytopsin preparations stained with the Kwik-Diff staining kit by counting 200 cells/slide.

2.5. Histopathological assessment

Immediately following euthanasia, lungs were isolated. The right lung was immediately frozen in liquid nitrogen and pulverized, then stored at -80 °C for further use. The left lung was filled with 4% paraformaldehyde for 24 h. The tissues were dehydrated with series of graded ethanol, and then embedded in paraffin wax. The blocks were made using paraffin and sections of 5 μ m thickness were cut for staining. The sections were stained with Hematoxylin and Eosin (H&E) to detect inflammatory infiltrates and vascular congestion, while Periodic Acid Schiff (PAS) was used to evaluate the goblet cell hyperplasia. Lungs were microscopically evaluated for inflammatory cell infiltration and

hyperplasia of goblet cells, as described previously [15,16]. The degree of goblet cell hyperplasia and inflammatory cell infiltration was scored by blind independent histopathologist.

2.6. Cytokines assay

The levels of IL-4, IL-5, IL-13, IL-10, IL-17A and IFN γ in BALF were measured using ELISA kits following to the manufacturer's instructions. The absorbance was read at 450 nm. Sample levels were assessed from standard curves, respectively.

2.7. Quantitative real-time PCR

Total RNA was extracted from homogenized lung tissues using Eastep® Super Total RNA Extraction Kit (Promega, Ltd, Shanghai, China) according to the manufacturer's instructions. 100 ng of complementary (c) DNA of each sample was used for later polymerase chain reaction (PCR), as described previously [17]. PCR primers were shown in Table 1. Reactions were run in duplicate for each sample and quantified in the ViiA 7 real-time PCR system (Applied Biosystems). A melting curve analysis was performed to control for the specificity of the amplification products. The expression of target genes was calculated by the $2^{-\Delta\Delta CT}$ method, and normalized to GAPDH values.

2.8. ROS Measurement in lung tissues

The relative changes of ROS in fresh lung tissue homogenates were measured by DCFH-DA method. The experiment was carried out within 1 h after tissue collection in buffer containing 130 mM KCl, 20 mM NaH₂PO₄, 20 mM Tris-HCl, 5 mM MgCl₂, and 30 mM glucose, plus 2 mM pyruvate and 2 mM malate (pH 7.4). The homogenates were diluted 500 times and incubated with DCFH-DA in buffer at a final concentration of 10 μ M at 37 °C for 15 min. The fluorescence intensity of DCF was recorded at the excitation wavelength of 495 nm and emission wavelength of 535 nm by using a fluorescent microscope (Nikon). The values were corrected by the value obtained by auto-oxidation of DCFH-DA in buffer only. Finally, the values were presented as relative fluorescence intensity compared with control group.

2.9. NAC treatment

NAC, the precursor of L-cysteine and reduced glutathione, not only acts as a direct scavenger of ROS but also has an indirect antioxidant effect by synthesizing glutathione *in vivo* [18]. NAC was dissolved in PBS

and was neutralized to the pH 7.4 with 1 M bicarbonate. The mice of NAC groups were treated intraperitoneally with NAC (150 mg/kg of body weight) 1 h before each OVA challenge, while PBS was injected to the mice of OVA groups. On day 28, animals were euthanized and lung tissues were collected for the experimental analyses.

2.10. Statistical analysis

All statistics were performed using SPSS 20.0 software. Measurement data were represented as mean $\pm$ SEM. Comparison of the mean between groups was performed by *t* test. *P* values < 0.05 were considered significant. All experiments were performed at least three times.

3. Results

3.1. Hv1-deficiency increases OVA-induced inflammatory cell infiltration in BALF

The migration of inflammatory cells into the lung is the main reason for causing allergic airway inflammation. In order to explore the effect of Hv1-deficiency on the migration of inflammatory cells, the BALF was collected 24 h after the last OVA challenge (Fig. 1A) and the total cells and differentiated cells including eosinophils, macrophages, neutrophils and lymphocytes were counted. As shown in Fig. 1B, the levels of all inflammatory cells in BALF were markedly increased in WTO and KOO mice compared with the corresponding normal control groups (WTC and KOC). In addition, compared with WTO mice, the number of neutrophils, eosinophils and total cells in BALF were markedly increased in KOO mice. But there were no obvious differences in the number of macrophages or lymphocytes in BALF between KOO and WTO mice. This result indicated that Hv1-deficiency exacerbated the recruitment of inflammatory cells into airway.

3.2. Hv1-deficiency aggravates OVA-induced inflammatory cell infiltration and goblet cell hyperplasia in lung tissues

Because Hv1 is related to the migration of immune cells, we next assessed the role of Hv1 in OVA-induced asthma in mice. The lung tissues were collected 24 h after the final aerosol OVA challenge and the histopathologic sections were performed to intuitively observe changes in lung tissues. PAS and H&E staining were performed on the lung tissues and the degrees of goblet cell proliferation and inflammatory cell infiltration were evaluated. As shown in Fig. 2A and C, compared with WTC and KOC mice, the WTO and KOO mice showed a marked infiltration of inflammatory cells around the bronchi and blood vessels of lung tissues, especially lymphocytes and eosinophils. Furthermore, KOO mice had a significant increase in the degree of infiltration of inflammatory cells around the bronchi and blood vessels of lung tissues compared with WTO mice. In addition, as shown in Fig. 2B and D, compared with WTC and KOC mice, PAS staining was positive in bronchial epithelium tissues of WTO and KOO mice. And compared with WTO mice, there were significant increases in goblet cell proliferation and mucus secretion in KOO mice. These results indicated that Hv1-deficiency aggravated OVA-induced lung tissues inflammation and goblet cell proliferation.

3.3. Hv1 deficiency leads to OVA-induced imbalance in Th1/Th2 and Treg/Th17 cytokine ratios

The imbalances in the ratios of Th1/Th2 and Treg/Th17 cytokines play an important role in the pathogenesis of allergic asthma. In order to determine whether Hv1-deficiency affects the balances in Th1/Th2 and Treg/Th17 cytokine ratios in BALF, IL-4, IL-5, IL-13, IL-10, IL-17A and IFN γ were measured by ELISA 24 h after the final challenge. The levels of IL-4, IL-5 and IL-13 were significantly increased in KOO and WTO mice compared with KOC and WTC mice (Fig. 3A-C). And compared with the

Table 1
Primer sequences along with product size.

Gene		Primer sequences	Product length
IL-4	Forward	ACGGAGATGGATGTGCCAAAC	83
	Reverse	AGCACCTTGAAGCCCTACAGA	
IL-5	Forward	AGCAATGAGACGATGAGGCTT	110
	Reverse	CCCCACGGACAGTTTGATT	
IL-13	Forward	TGTGTCTCTCCCTCTGACCC	128
	Reverse	CTACACAGAACCCGCCAGC	
Hv1	Forward	TGATCTTGCTTCGGCTCTGG	166
	Reverse	ATTTCTTGTCTCTCTCGGAGC	
NOX2	Forward	GGGAACCTGGGCTGTGAATGA	147
	Reverse	CAGTGCTGACCCAAGGAGTT	
NOX4	Forward	TACTACATTACCAAATGTTGGGC	146
	Reverse	CTGCTAGGGACCTTCTGTGAT	
DUOX1	Forward	AGGATGCTAAGTCTTCATCG	211
	Reverse	TGATCACTTCCGGCACTTCC	
Catalase	Forward	CTCGCAGAGACCTGATGTCC	286
	Reverse	TGTGGAGAATCGAACGGCAA	
SOD2	Forward	GAACAATCTCAACGCCACCG	179
	Reverse	CCAGCAACTCTCTTTGGGTT	
GAPDH	Forward	CTCATGACCACAGTCCATGCCATCACTG	465
	Reverse	CATGAGGTCCACCACCTGTTGCTGTA	

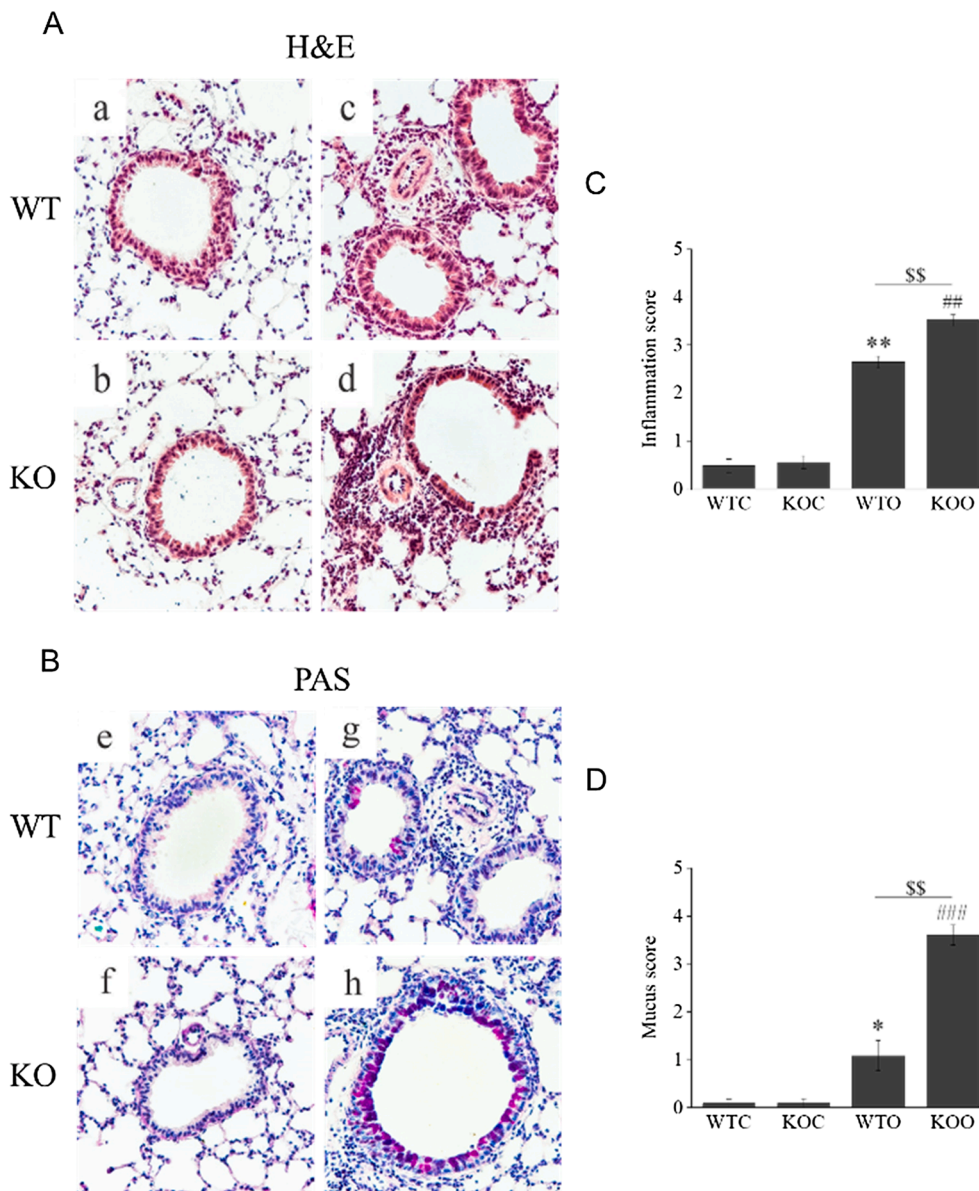


Fig. 2. Effect of Hv1-deficiency on histopathologic changes in the lung tissues of OVA-challenged asthma mice. (A, B) The lung tissues were stained with H&E (A) for histopathological examination and PAS (B) for goblet cells proliferation. Lung tissues of normal groups and OVA groups were obtained 24 h after the final challenge. (C) Scoring of inflammatory cell infiltration. Values are expressed as mean $\pm$ SEM ($n = 6$ for each group). ** $P < 0.01$ vs. WTC; ## $P < 0.01$ vs. KOC; \$\$ $P < 0.01$ vs. WTO. (D) Scoring of goblet cell proliferation. Values are expressed as mean $\pm$ SEM. * $P < 0.05$ vs. WTC; ### $P < 0.001$ vs. KOC; \$\$ $P < 0.01$ vs. WTO.

WTO mice, the levels of IL-4 and IL-13 in BALF of KOO mice were observably increased. Meanwhile, we collected lung tissues to evaluate gene expressions of Th2 cytokines. The rt-qPCR analysis showed the same results. The expressions of OVA-specific Th2 cytokines were markedly increased in OVA groups. And they all had statistical difference between WTO mice and KOO mice (Fig. 3D-F). Moreover, the levels of IFN γ and IL-10 were obviously decreased, but IL-17A was greatly increased in KOO and WTO mice compared with that in control mice. And compared with WTO mice, the secretions of cytokine IFN γ by Th1 cells and anti-inflammatory cytokine IL-10 by Treg cells in KOO BALF were dramatically decreased. The secretion of inflammatory factor IL-17A by Th17 cells in KOO BALF was substantially higher compared to that in WTO mice (Fig. 3G-I). These results revealed that Hv1 may participate in the process of airway inflammation injury in asthma by regulating Th1/Th2 and Th17/Treg immune balance.

3.4. Hv1-deficiency increases OVA-induced NADPH oxidase genes expression and decreases anti-oxidase genes expression

ROS is closely related to the pathogenesis of asthma. In order to further evaluate the effect of Hv1 on asthmatic mice, we measured the

expressions of Hv1 and proteins related to ROS production in lung tissues by rt-qPCR. Hv1 mRNA expression was significantly up-regulated in WTO mice as compared with WTC mice (Fig. 4A). Moreover, the mRNA levels of NOX2, NOX4 and DUOX1 that play important roles in allergic airway inflammation and mucus secretion [19], were markedly increased in KOO and WTO mice compared with KOC and WTC mice. And the mRNA levels of NOX2, NOX4 and DUOX1 were observably increased in KOO mice compared with WTO mice (Fig. 4B-D). However, the mRNA levels of SOD2 and catalase were dramatically decreased in OVA mice compared with control mice, and they were significantly decreased in KOO mice compared with WTO mice (Fig. 4E-F). These results might suggest that Hv1-deficiency results in a huge increasing in ROS production due to the reduction in SOD2 and catalase and the increasing in NOX2, NOX4 and DUOX1.

3.5. Hv1-deficiency aggravates OVA-induced ROS production in lung tissues

Under physiological conditions, ROS can act as the second messenger to activate multiple signaling pathways, which can regulate cell growth, proliferation and differentiation. However, excessive ROS can cause the

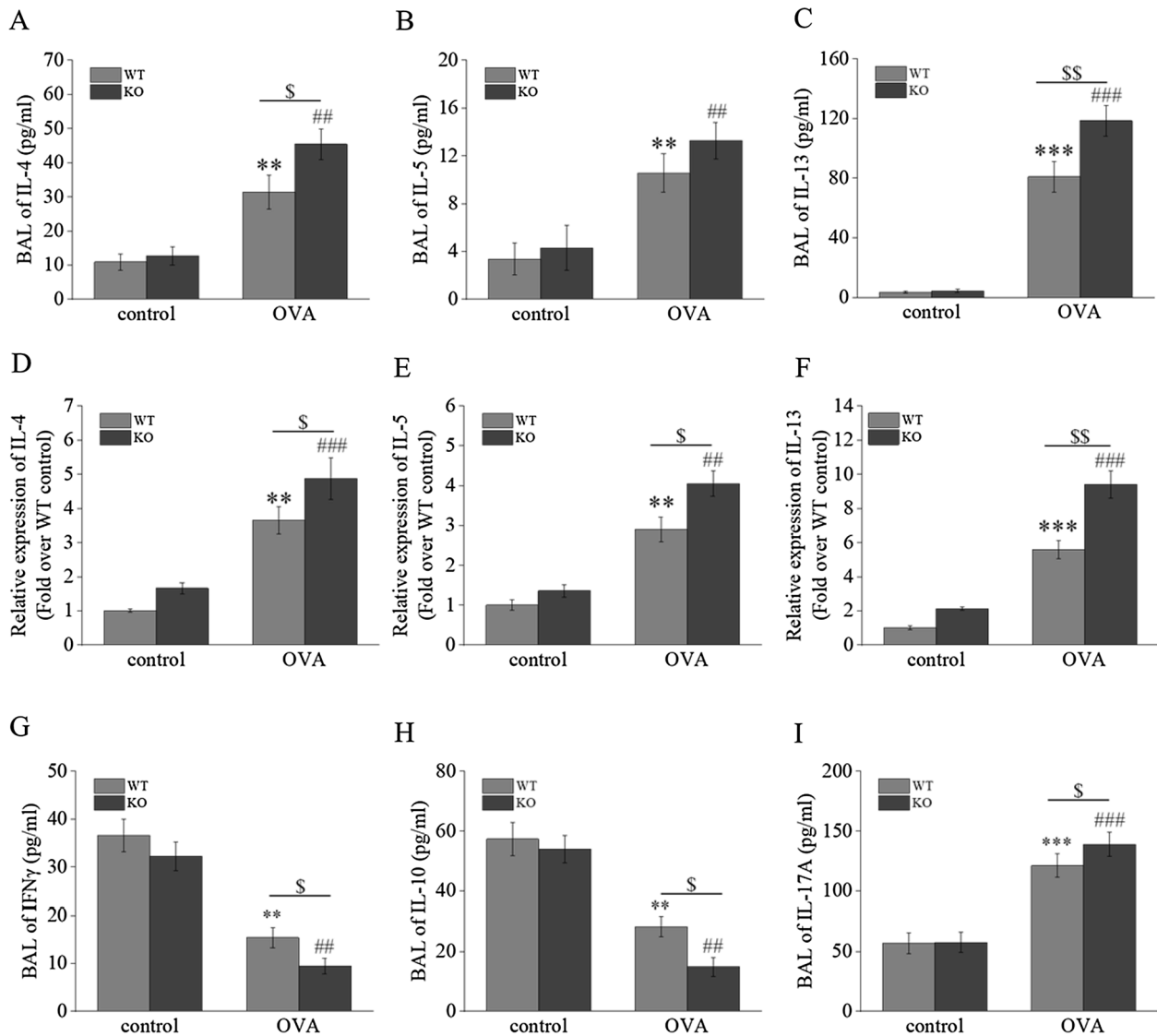


Fig. 3. Effect of Hv1-deficiency on the expression levels of IL-4, IL-5, IL-13, IFN γ , IL-10 and IL-17A. (A, B, C) The levels of IL-4 (A), IL-5 (B) and IL-13 (C) were measured by ELISA. (D, E, F) The gene expressions of IL-4 (D), IL-5 (E) and IL-13 (F) were detected by rt-qPCR. (G, H, I) The levels of IFN γ (G), IL-10 (H) and IL-17A (I) were measured by ELISA. BALF was collected 24 h after the last challenge. Lung tissues were collected 24 h after the last challenge. GAPDH was used as an internal control. Values are shown as mean $\pm$ SEM (n = 6 for each group). ** P < 0.01, *** P < 0.001 vs. WTC; ## P < 0.01, ### P < 0.001 vs. KOC; \$ P < 0.05, \$\$ P < 0.01 vs. WTC.

activation of signaling pathways which are related to oxidative stress, pro-apoptosis, and pro-fibrosis, and then induce asthma. In order to explore the effect of Hv1-deficiency on ROS production in lung tissues, fresh lung tissue homogenates were collected 24 h after the last OVA challenge and the production of ROS were detected by DCFH-DA method. As shown in Fig. 4G, the ROS production in the lung tissue homogenates were obviously increased in WTC and KOC mice compared with that in WT and KO mice. Moreover, the ROS production in lung homogenates of KOC mice was significantly increased compared with WTC mice. These results suggested that Hv1-deficiency induced oxidative stress in lung tissues by increasing OVA-induced overproduction of ROS.

3.6. OVA-induced asthma in Hv1-deficient mice can be reversed by NAC

NAC, a ROS scavenger, is a mucolytic and anti-inflammatory drug, which has beneficial effects on the alleviation of allergic lung inflammation [20]. As shown in Fig. 5A–D, NAC treatment led to an obvious decrease not only in inflammatory cell infiltration but also in goblet cell

hyperplasia in lung tissues compared with OVA-challenged mice. In addition, the NAC treatment markedly decreased the level of ROS in lung homogenates of KO mice (Fig. 5E), indicating that the exacerbated asthma induced by OVA in Hv1-deficient mice is due to excessive ROS. Our data demonstrated that NAC treatment can decrease the infiltration of inflammatory cell, the hyperplasia of goblet cells, and the production of ROS.

4. Discussion

In present work, our data showed that knockout of Hv1 aggravates OVA-induced allergic lung asthma in mice, accompanied with upregulations of inflammatory factors, cytokines, NOX2, NOX4 and DUOX1, and downregulations of SOD2 and catalase in lung tissue. Treatment with NAC significantly reduces the ROS production in lung of Hv1-deficient mice. To our knowledge, it is the first time to investigate the anti-inflammatory effects of Hv1 in a mouse model of allergy asthma. Our results suggested that Hv1 might be as a potential target for the development of drugs to treat asthma.

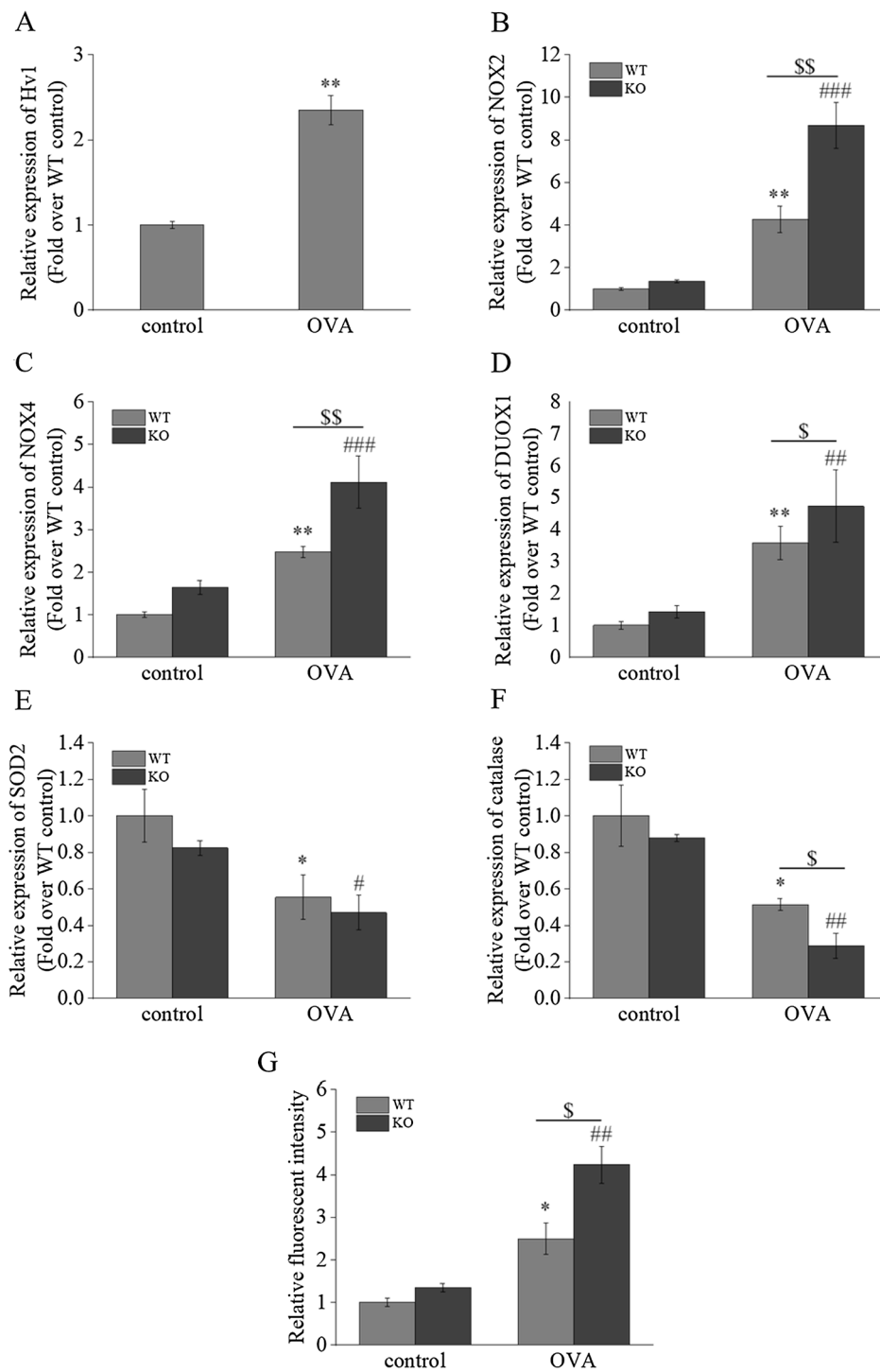


Fig. 4. Effect of Hv1-deficiency on the expression levels of NOXs. (A-F) The gene expressions of Hv1 (A), NOX2 (B), NOX4 (C), DUOX1 (D), SOD2 (E) and catalase (F) were detected by rt-qPCR. GAPDH was used as an internal control. Lung tissues were collected 24 h after the last challenge. (G) ROS production in lung tissues were detected 24 h after the last challenge. Values are shown as mean $\pm$ SEM (n = 6 for each group). * P < 0.05, ** P < 0.01 vs. WTC; ## P < 0.01, ### P < 0.001 vs. KOC; \$ P < 0.05, \$\$ P < 0.01 vs. WTC.

Allergic asthma is one of the most common chronic diseases affecting the airways of children and adults. Allergens induce the recruitment of a variety of inflammatory cells, cytokines and inflammatory mediators that cause airway inflammation [21]. The clinical manifestations of the patients are sneezing, runny nose, cough, chest distress and other symptoms. Although the pathophysiology of allergic asthma has been widely studied, most modern drugs have only transiently improved clinical symptoms and there is still no cure for this disease [22].

It has been reported that allergic asthma is closely related to the imbalance of Th1/Th2 cells and their specific cytokines. Under physiological conditions, the immune responses of Th1 and Th2 cells maintain a dynamic balance. Once this balance is broken, diseases will occur [21].

The Th1 cytokine IFN γ is associated with antigen-induced eosinophilic infiltration and airway hyperresponsiveness. Th2 cytokines have been demonstrated to play an important role in the pathogenesis of allergic airway inflammation both in animals and human. For example, IL-5 is the key cytokine for activation and survival of eosinophils, while IL-13 is the key cytokine for eosinophil infiltration into lung tissues [1]. And IL-4 plays an important role in the development of allergic inflammation by promoting the differentiation of Th0 cells in favor of Th2 cells, promoting the release of cytokines, mucus secretion, IgE isotype switching and transmigration of eosinophils across endothelium [3]. Recent study showed that Treg/Th17 immune imbalance is also one of the important mechanisms in the pathogenesis of asthma [23]. The cytokines IL-10 and

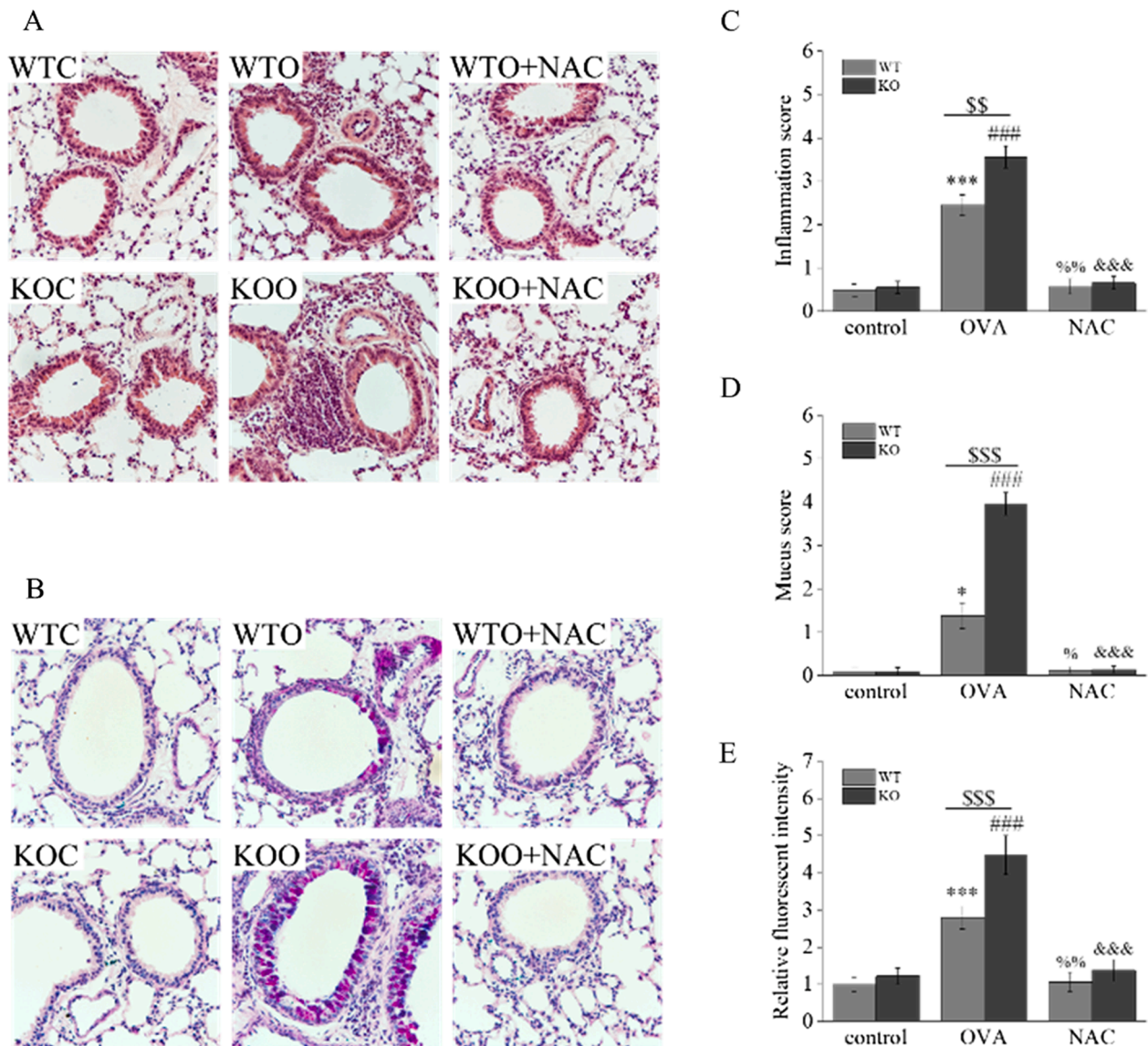


Fig. 5. Treatment with NAC alleviates OVA-induced asthma in Hv1-deficient mice through removal of ROS. The lung tissues were stained with H&E (A) and PAS (B). (C) Scoring of inflammatory cell infiltration. (D) Scoring of goblet cell proliferation. (E) ROS production in lung tissues. Lung tissues were collected 24 h after the last challenge. Values are shown as mean $\pm$ SEM (n = 6 for each group). * P < 0.05, *** P < 0.001 vs. WTC; #### P < 0.001 vs. KOC; % P < 0.05, %% P < 0.01 vs. WTO; &&& P < 0.001 vs. KOO.

TGF- β 1 secreted by Treg cells can regulate the immune balance and anti-inflammation, and the cytokine IL-17A released by Th17 cells can promote the activation of airway epithelial cells, smooth muscle cells and fibroblasts. Meanwhile, IL-17A can enhance the release of inflammatory factors by multiple cells and mucus by the mucous glands, recruit neutrophils and strengthen the airway hyperresponsiveness. In the present work, we showed that there are significant increases in the levels of IL-4, IL-5, IL-13 and IL-17A, and marked decreases in IFN γ and IL-10 in BALF of KOO and WTO mice. These results suggested that Hv1 deficiency may aggravate asthmatic airway inflammation through the imbalance of Th1/Th2 and Th17/Treg cell ratios.

Hv1 channel has been detected in many cell types, including macrophages, blood cells, airway epithelia, sperm and microglia [24]. Hv1 channel is associated with the charge compensation and acid extrusion during the oxidative burst [25], pH regulation of alveolar epithelium [26], maintenance of eosinophil activity, neutrophils calcium entry, activation of B cells during immune response [27], and histamine release by basophils [28]. During asthma, increased acid secretion into the alveoli is thought to be a contributing factor to the inflammation of

lung tissue [29]. The higher expression level of Hv1 in lung tissues of WTO mice, in the present work, might suggest that Hv1 plays an important role in asthma. The lacking of Hv1 might affect maintaining the pH of airway surface liquid (ASL) at a mildly acidic pH for normal physiology, and contribute to disease.

The migration of eosinophils to the lung is the main reason for causing allergic airway inflammation, which can regulate immune response, cause airway hyperresponsiveness, airway remodeling, epithelial cell damage and mucus hypersecretion [30]. Many studies have shown that Hv1 is expressed at a high level in mouse eosinophils, which does not affect migration of mouse eosinophil, and is required for preventing the activation of eosinophils [31]. In our present study, knockout of Hv1 remarkably promotes the infiltration of eosinophil in mouse lung tissues. Our results suggested that Hv1 deficiency may cause the sharp increase in OVA-induced eosinophilic infiltration, which leads to obvious goblet cell proliferation and mucus production in the lungs of Hv1 KO mice.

ROS play a vital role in human physiological and pathophysiological processes, not only regulating host defense, but also participating in

inflammation and immune responses [7]. ROS produced by eosinophils, neutrophils and T cells have been shown to play an important role in the pathogenesis of asthma. Lots of studies have delineated that Hv1 maintains a high level of ROS generation during NOX activity through charge compensation and acid extrusion in the leukocyte respiratory burst, and absence or inhibition of Hv1 results in excessive depolarization and cytoplasmic acidification [25,32]. Surprisingly, some studies have reported that the genetic ablation of Hv1 increases basal ROS production and ROS production induced by low doses of N-formyl-Met-Leu-Phe (fMLF) in neutrophils [33,34]. Hv1 also exerts suppressive effects on ROS production in microglia, which is largely enhanced in primary cultures of Hv1 KO microglia than in WT microglia [35,36]. In our previous work, the high expression levels of NOX4 and NOX2 in adipocytes in Hv1 KO mice when challenged with nutrition, proposed that loss of Hv1 leads to the increased ROS production in adipocyte [37]. It has been reported that in the absence of Hv1 or with inhibition of its channel function, the ability of the NOX2 system to catalyze sustained generation of microbicidal ROS decreases by 30–75%, and the bacterial killing by neutrophils can be impaired [38]. In addition, Okochi et al. [39] have demonstrated that activated Hv1 inhibits the exocytosis of azurophilic granule enzymes in neutrophils, including MPO, thereby reducing the generation of microbicidal HOCl. They used the *Candida albicans* pneumonia model found that although the viable organisms from lung tissue can be eliminated effectively in Hv1 KO mice, they exhibited more severe pyogenic granulomatous inflammation than WT mice. In present work, we showed that the gene expressions of NOX2, NOX4 and DUOX1 are increased in lung tissues of Hv1 KO mice, whereas SOD and catalase are decreased, indicating that loss of Hv1 maintains high levels of ROS in lung tissues when challenged with OVA and then aggravates allergic asthma. Therefore, the lack of Hv1 may be involved in the increased production of HOCl in neutrophils after challenged with OVA, and excessive HOCl may damage the host defense response, leading to more severe pulmonary inflammation.

Histamine is an important chemical mediator of inflammation in asthma [40]. Musset et al. [28] showed that Hv1 is highly expressed in basophils, and inhibiting Hv1 by zinc *in vitro* reduces histamine release from basophils. It seems to be paradoxical to that Hv1 KO exacerbates asthma in the present work. In fact, histamine is stored in many kinds of cells, such as mast cells, basophils, platelets, histaminergic neurons, and enterochromaffin cells [40], suggesting that Hv1 KO may not affect the histamine release in asthma. It is well known that Hv1-deficient B cells have high levels of ROS and protein tyrosine phosphatase SHP-1 oxidation [41], which affects downstream antibody-stimulated histamine release and then aggravates asthma.

Taken together, the functions of Hv1 in airway epithelia, macrophages and blood cells contribute to our better understanding of the potential role of Hv1 in asthma and also provide new research directions for asthma treatment with Hv1 as a drug target.

5. Conclusions

In conclusion, our data showed that the mice deleted Hv1 do not display an overt phenotype under normal conditions but may have a latent tendency of autoimmune diseases, thus showing the exacerbation of asthma after OVA challenged. This process may be associated with the high levels of NOXs expression, low levels of antioxidant enzymes expression and abnormal production of ROS in lung tissues, which may increase inflammatory cell infiltration, goblet cell metaplasia and Th2 cytokines level. And this process can be reverse by antioxidant, such as NAC.

Author contributions

HYD and SJL conceived and designed the study. HYD, HMP, YTG and YFZ performed the experiments. HYD and SJL wrote the paper. SJL and HYD reviewed and edited the manuscript. All authors were involved in

data analysis, read and approved the manuscript.

CRedit authorship contribution statement

Hongyan Du: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Writing - review & editing. **Huimin Pang:** Validation, Formal analysis, Investigation. **Yingtang Gao:** Validation, Formal analysis, Investigation, Resources. **Yongfa Zhou:** Formal analysis, Investigation. **Shu Jie Li:** Conceptualization, Methodology, Resources, Writing - original draft, Writing - review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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