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# 5-BDBD ameliorates an OVA-induced allergic asthma by the reduction of Th2 cytokines production

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**ABSTRACT**

***Objective(s)***: P2X4R is expressed in immunocyte and lung tissues. It has been a focus in inflammatory responses recently. This study investigated whether blockage of P2X4R attenuates allergic inflammation by modulating T cell response in ovalbumin-sensitized mice.
***Materials and Methods:*** Ovalbumin was used to sensitize and challenge for a mouse model. Intranasal application of 5-BDBD, P2X4R antagonist, were performed 3 hr before each airway allergen challenge. The lung was evaluated for P2X4R by real-time PCR and immunofluorescence. Th1/Th2 cytokines in bronchoalveolar lavage fluid were measured by ELISA. T-bet, Gata-3, and p-p38 MAPK were measured by Western blot or real-time PCR.
***Results:*** P2X4R was overexpressed in the lung after allergen challenge compared with the control group. Blockage of P2X4R decreased inflammation in the lung, IL-4 expression was reduced as well as IL-5; IFN-γ expression was elevated in BALF in ovalbumin-sensitized mice. Moreover, blockage of P2X4R inhibited ovalbumin-induced increased Gata-3 level and decreased T-bet level.
***Conclusion:*** These findings suggest that 5-BDBD ameliorates an ovalbumin-induced asthmatic attack by the downregulation of cytokines related to the Th2 cell.

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## Introduction

The typical characteristics of anaphylactic asthma are chronic inflammation and remodeling of the airway (1, 2). The balance of helper T cell 1 (Th1) / helper T cell 2 (Th2) is crucial for the maintenance of immune homeostasis. Th2 responses are associated with the differentiation of T lymphocytes and the recruitment of eosinophils. Cytokines secreted by allergen-specific type 2 T-helper cells are increasingly recognized to have the key role in chronic airway inflammation in asthma (3). IL-4 is essential for IgE switching of B lymphocytes, and IL-5 selectively acts on eosinophil maturation, survival, and activation (4). IgE level in BALF (bronchoalveolar lavage fluid) is closely related to IL-4 and IL-5 secretion. The increase of IL-4 and IL-5 indicates the occurrence of Th2 inflammation in asthma (5). Th1-type cytokine and interferon gamma (IFN-γ) can activate macrophages, which play a role in immune response (6, 7). T-bet and GATA-3 are transcription factors found in recent years, which is a specific transcription factor for inducing the polarity of Th1/Th2 and secreting the effector cytokines. It was concluded that the T-bet/GATA-3 expression could indirectly reflect the proportion of Th1 and Th2 cells (8). Transcription factors regulate the secretion of cell cytokines and inflammation cytokines in the transcription level, which is the hot topic for studying the pathogenesis of asthma (9).

It is well known that P2 purinergic receptors can be activated by extracellular ATP, which is a red light for hinting the initiation of the immunologic process in disease (10). P2 purinergic receptors include P2XR (P2X1-7) and P2YR (P2Y 1–14). P2YR belongs to G-protein–coupled receptors, P2XR belongs to ligand-gated ion channels (11-13). ATP levels are increased in patients with asthma and in ovalbumin (OVA)-sensitized mice. Endogenous or exogenous ATP could aggravate the reaction of Th2 to OVA. Furthermore, P2X4R has become a focus in inflammatory responses recently. P2X4R is expressed in immunocyte and lung tissues such as alveolar, lymphocytes, and so on (14-16). ATP-mediated P2X4R signaling pathway plays a role in inflammatory response by regulating IL-1 beta; IL-6 and TNF-α secrete in peripheral nerve injury (17). Inhibition of P2X4R attenuated the inflammation and damage in collagen-induced arthritis (18, 19). Blockade of P2X4Rs-p38 MAPK pathway in the spinal cord may alleviate neuropathic pain (20). Thus, we hypothesized that blockade of P2X4R may alleviate airway inflammation in allergic asthma in mice.

In this research, it is proposed that P2X4R antagonist, 5-BDBD, inhibits inflammation cytokines and affects ratio of T-bet/Gata-3. Furthermore it clarifies whether 5-BDBD inhibits allergic inflammation by modulating T cell response in OVA-sensitized mice.

## Materials and Methods

### Chemicals and reagents

We bought the primary antibodies and the secondary antibodies from Santa Cruz Biotechnology Inc. (Santa Cruz, California, USA). ELISA kits were purchased from Boster (Wuhan, China). We bought 5-BDBD from Tocris Bioscience (Bristol, UK), Grade V OVA from Sigma-Aldrich Corp. (St Louis, Missouri). The TRIzol reagent and the SYBR Green system was purchased from

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TAKARA Bio Inc (Dalian, China). We obtained the other common reagents through common means.

***Sensitization and airway challenge***

We obtained BALB/c mice from the Laboratory Animal Research Center in Beijing, China. Mice were female and 6-8 weeks old. They were raised pathogen-free, provided with 12 hr light-dark cycle, and given food and water at room temperature. Mice were divided into 4 groups (n=7): the phosphate-buffered saline (PBS) control group, the control+5-BDBD group, the OVA group, and the OVA+5-BDBD group. Mice of the OVA group were injected with 20 μg of OVA mixed with 2.0 mg of aluminum hydroxide for sensitization by intraperitoneal injection on the 1$^{st}$ day. They were injected with 10 μg of OVA mixed with 1.0 mg of aluminum hydroxide for sensitization by intraperitoneal injection on the 8$^{th}$ day, the 15$^{th}$ day, and the 22$^{nd}$ day. Beginning on the 23$^{rd}$ day, 4% OVA in PBS was used for challenging the mice for about 25 to 30 min daily, which lasted for 7 days on the basis of the methods of Choi *et al.* (21) and Vanacker *et al.* (22) with some modifications. We performed the same method for sensitizing and challenging in the OVA+5-BDBD group, but mice underwent 5-BDBD (30 μmol) (23) 3 hr before each airway challenge by intranasal administration. In the control group, mice were sensitized and challenged with PBS instead of OVA, and mice in the control+5-BDBD group underwent intranasal application of 5-BDBD (30 μmol) 3 hr before each challenge with PBS.

***Real-time PCR for P2X4R mRNA, Gata-3 mRNA, and T-bet mRNA expression***

Extraction of total RNA in the lung was done with TRIzol reagent (TRIzol reagent, TAKARA Bio Inc.) in accordance with the specification, then we conducted reverse transcription polymerase chain reaction on the complementary DNA samples using the SYBR Green system (TAKARA Bio Inc.). Mouse TATA-binding protein (TBP) was used as an endogenous control for gene expression normalization. The fold changes were calculated using the ΔΔCt method of relative comparison. The sequence for the primer sets used is as follows. The primers used were P2X4R sense 5'-ATCGTCACCGTGAACCAGAC-3' and P2X4R antisense 5'-GCGTCTGAATCGCAAATGCT-3', GATA-3 sense 5'-CTTATCAAGCCCAAGCGAAG-3' and GATA-3 antisense 5'-CCCATTAGCGTTCCTCCTC-3', T-bet sense 5'-TCAACCAGCACCAGACAGAG-3' and T-bet antisense 5'-AACATCCTGTAAT GGCTTGTG-3', TBP sense 5'-GTGGATCGAGTCCGGTA GC-3', TBP antisense 5'-AAT AGTGATGCTGGGCACTG-3'. Cycle and threshold were obtained in accordance with the specification of the manufacturer.

***Immunofluorescence detection of P2X4R***

Lungs were fixed in 4% paraformaldehyde, 30% sucrose dehydration, embedded in OCT (Opti-Mum Cutting Temperature Compound), and sectioned at 4 μm thickness. Using cold acetone, frozen sections from the PBS control and the OVA group were fixed for 10 min, then incubated by rabbit polyclonal anti-P2X4R primary antibodies (1:50 dilution, 0.01mol/L PBS, pH 7.4) overnight at 4 °C. Next, rinsed thrice with PBS, incubated with goat anti-rabbit IgG conjugated to fluorescein isothiocyanate (Santa Cruz Biotechnology) at 1:200, the incubation was at 37 °C for 30 min in the dark. Slides were then mounted with glycerol and images captured using a fluorescence microscope.

***Histological examination in the lungs***

In order to make the pleura extend and flatten, the collected lung tissues were perfused with 4% paraformaldehyde. Next, the lung tissues were fixed in 4% paraformaldehyde. Paraffin was applied for routine embedding, then sectioned with 5 μm. At last, hematoxylin and eosin (HE) staining was done. These tissues were scored for inflammatory cells based on this principle, which was absent (0), rare (1), mild (2), moderate (3), or severe (4) by an observer blinded to the experimental groups.

***Analysis of Th1 and Th2 cytokines by ELISA***

BALF was obtained as previously described (24, 25). The concentrations of IL-4, IL-5, and IFN-γ in BALF were detected with ELISA kits by using the protocols (Boster, Wuhan, China). The sensitivity of these assays to IL-4, IL-5, and IFN-γ was lower than 1 pg/ml, 2 pg/ml, and 2 pg/ml, respectively.

***Western Blot for T-bet and Gata-3, and phosphated-p38 MAPK***

Protein levels were assessed by western blot in lung tissues. Using RIPA lysis buffer obtained from Beyotime in China, 0.05 g lung tissues were homogenized. Protein quantification was done with Enhanced BCA Protein Assay Kit (Beyotime). Equal amounts of lysate proteins were detached using SDS-PAGE, then transferred to nitrocellulose membranes, which were blocked for 1 hr by 5% nonfat milk in 0.1% TBS/Tween. Next, the membranes were incubated with the respective primary antibodies against phosphated-p38 MAPK, T-bet, or Gata-3 overnight at 4 °C. Binding of secondary HRP antibodies was performed. ECL enhanced chemiluminescence method was applied to examine the proteins. Equal loading of protein was confirmed by the Western bolt for β-actin or the total protein.

***Statistical analysis***

Data were analyzed as means ± SE. Statistical analysis was done by Student's t-test and one-way analysis of variance (ANOVA). The difference was considered to be statistically significant at $P<0.05$.

## Results

***Expression of P2X4R in lung tissue***

Expression of P2X4R in lungs was studied by real-time PCR (Figure 1A). The results demonstrated that P2X4R was expressed in both the control and the OVA group. Moreover, compared with the control group, the level of P2X4R in the OVA group was significantly increased. Expression of P2X4R in lungs was also detected by immunofluorescence (Figure 1B). The green fluorescence emitted by the FITC-labeled P2X4R antibody indicated the expression of P2X4R in lungs. Nuclei were stained by Dapi in blue. The green

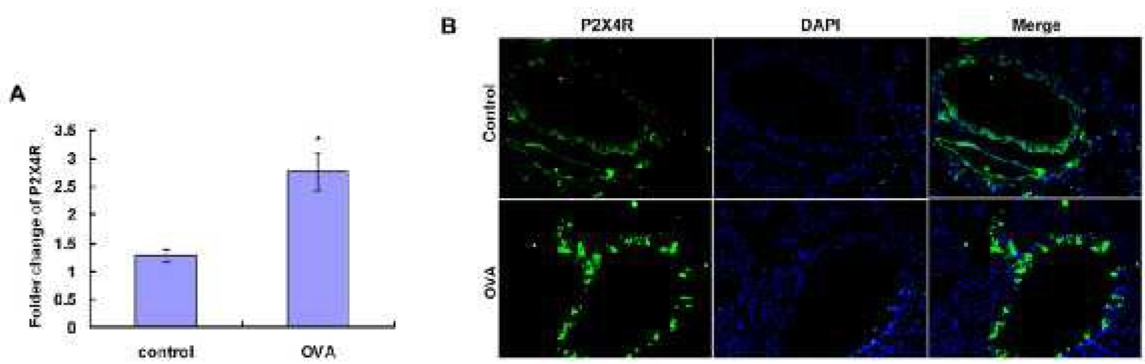


**Figure 1.** P2X4R contributes to allergic asthma. A: Real-time PCR was used to detect P2X4R expression in lung tissues. *$P$<0.05 versus control. B: Expression of P2X4R in the frozen section of the lung by immunofluorescence. Representative images are shown of P2X4R staining (green) and nuclei staining (in blue). The green fluorescence was clearly more intense in the OVA group than in the control group (original magnification, fluorescence microscopy, ×200

fluorescence in the OVA group was clearly more intense in comparison with the control group.

### *Effect of 5-BDBD on OVA-induced inflammation in BALF and lung tissues*

By histological analysis of the tissues, it was detected that inflammatory cells were widely infiltrated, surrounding the alveoli, bronchioles, and blood vessels in OVA-sensitized mice. It indicated the successful establishment of the asthmatic model. The infiltration of inflammatory cells was significantly more prevalent in untreated asthmatic mice than in those treated with 5-BDBD (Figure 2).

### *Effect of 5-BDBD on Th1 and Th2 cytokines in BALF by ELISA*

Effect of 5-BDBD on IL-4, IL-5, and IFN-γ was shown (Figures 3 A, B, C). Expression of IL-4 and IL-5, Th2

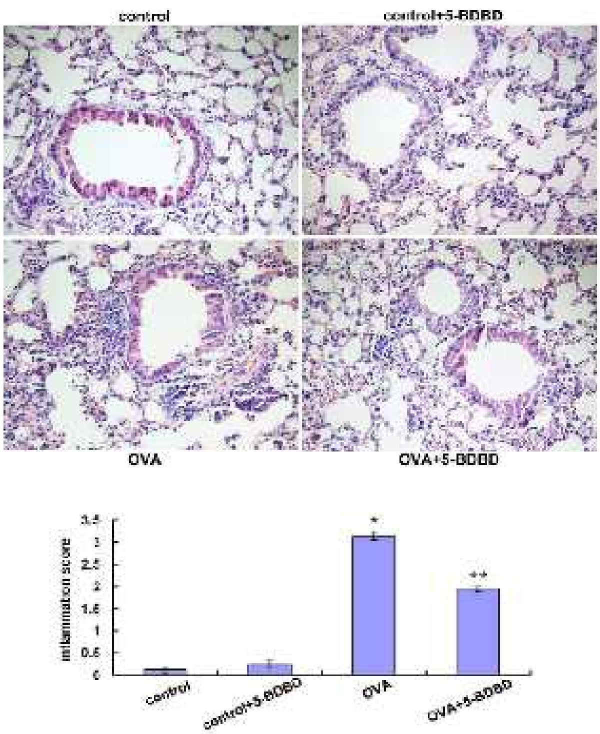


**Figure 2.** 5-BDBD alleviated ovalbumin-induced airway inflammation. HE staining was applied for detecting the pathological changes in the lung. (light microscopy, ×200). *$P$<0.01 versus control; **$P$<0.01 versus OVA

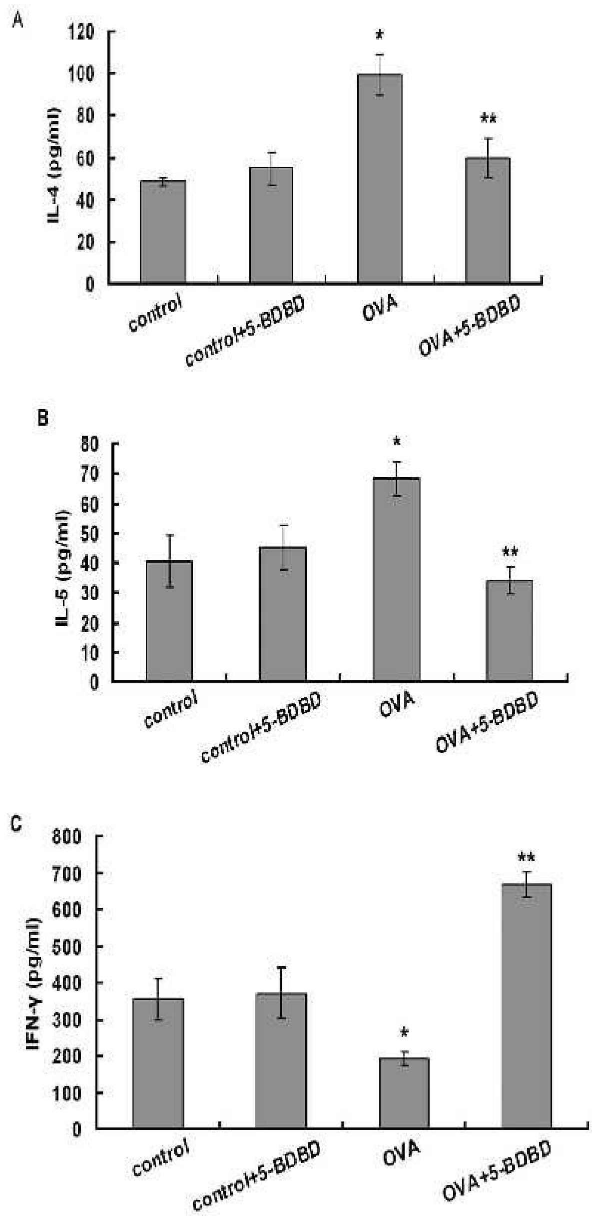


**Figure 3.** Effect of 5-BDBD treatment on the Th1/Th2 cytokines in the supernatant of bronchoalveolar lavage fluid. A: ELISA was used to detect the level of IL-4. *$P$<0.01 versus control; **$P$<0.01 versus OVA. B: ELISA was used to detect the level of IL-5. *$P$<0.05 versus control; **$P$<0.01 versus OVA. C: ELISA was used to detect the level of IFN-γ. Data are expressed as mean ± S.E. (n = 6). *$P$<0.05 versus control; **$P$<0.01 versus OVA

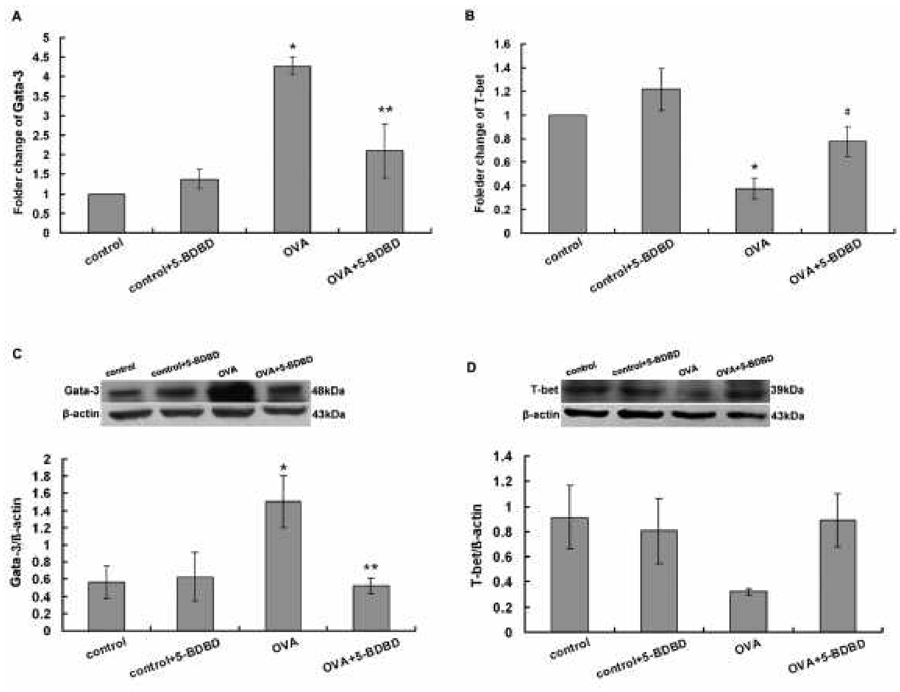


**Figure 4.** 5-BDBD alleviated inflammation via Gata-3/T-bet. A: Real-time PCR was used to detect Gata-3 expression in lung. **P*<0.01 versus control; ***P*<0.01 versus OVA. B: Real-time PCR was used to detected T-bet expression in lung. **P*<0.01 versus control; #*P*<0.05 versus OVA. C: Gata-3 expression in the lung by the Western blot. **P*<0.05 versus control; ***P*<0.05 versus OVA. D: T-bet expression in the lung by the Western blot. **P*<0.05 versus control; ***P*<0.05 versus OVA

typical cytokines in BALF of OVA-sensitized mice was significantly elevated, and IFN-γ was reduced, compared with those of the control mice. However, IL-4 and IL-5 expressions in BALF of OVA-sensitized mice were reduced by treatment with 5-BDBD, and IFN-γ level was increased by treatment with 5-BDBD. Moreover, the administration of 5-BDBD played no role in IL-4, IL-5, and IFN-γ expression in control mice.

### *Effect of 5-BDBD on T-bet/Gata-3 in lung tissue*

Expression of Th1/Th2-related T-bet/Gata-3 in mRNA of lungs was detected by real-time PCR, protein was detected by Western blot. As shown in (Figures 4 A, B, C, D), the level of Gata-3 in the OVA group was higher than that in the control group. Application of 5-BDBD decreased Gata-3 expression of OVA-sensitized mice. However, the level of T-bet in the model group was lower than the control group. Application of 5-BDBD increased T-bet expression of OVA-sensitized mice. But it was no significant in protein level.

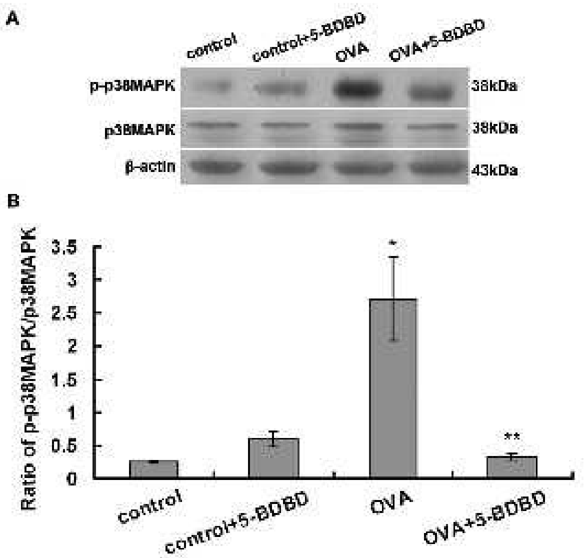


**Figure 5.** Effect of 5-BDBD on airway Th2 inflammation by p38 MAPK. A: Expression of p-p38 MAPK and p38 MAPK in lung tissues. B: P-p38 MAPK protein relative to p38 MAPK was analyzed statistically by optical density. **P*<0.05 versus control; ***P*<0.05 versus OVA

### *Expression of phosphated-p38 MAPK in lung tissue*

Western blot was applied to the measured expression of phosphated-p38 MAPK/p38 MAPK (Figures 5 A, B) in the lungs. The level of phosphated-p38 MAPK/p38 MAPK in the control mice was low, but it was significantly elevated in mice of the OVA group. However, 5-BDBD treatment significantly decreased the upregulation of phosphated-p38 MAPK/p38 MAPK in the OVA group. However, 5-BDBD treatment had no effect on p38 MAPK activation in the control mice.

## Discussion

Chronic inflammation, remodeling and hyperresponsiveness of airways are the typical characteristics of asthma. Moreover, T-helper 1 (Th1) and T-helper 2 (Th2) cells are closely related to airway inflammation (26). These inflammatory responses are attributed to Th2 cells, together with other inflammatory factors, including mast cells, B cells, eosinophils, cytokines, and chemokines (27). IL-4, IL-5, and IL-13 are the typical cytokines produced by Th2 cells, which play a role in initiation of Th2 inflammatory responses (28). Along with the increased secretion of Th2 cytokines, Th1 cytokines such as IFN-γ were reduced. Thl/Th2 imbalance causes a series of airway inflammations in allergic asthma. ATP has previously been shown to mediate allergen-driven lung inflammation (29). Usually, ATP plays its role by activating P2X and P2Y signaling. It has been proven that P2X4R is distributed in the lungs, kidneys, blood vessels, and immunocytes (30, 31).

Activated T cells by antigens can differentiate into different subsets of functional T cells such as Th1, Th2, Th17, and Treg cells, which is regulated by transcription factors of T-bet, Gata-3, RORγT, and Foxp3, respectively. Moreover, Thl/Th2 imbalance causes a series of airway inflammations is a classic theory in allergic asthma. In the present study, we have shown P2X4R level was increased in the OVA-sensitized mice in comparison with that in the control mice. We found significantly increased inflammatory cells infiltration in the OVA mice, that is, increased IL-4 and IL-5 levels, and decreased IFN-γ levels. But treatment with 5-BDBD dramatically decreased the inflammatory cells infiltration of OVA-sensitized mice. IL-4 and IL-5 expression of OVA-sensitized mice were decreased by 5-BDBD treatment, but IFN-γ expression of OVA-sensitized mice was increased by 5-BDBD treatment. Furthermore, we found treatment with 5-BDBD increased T-bet level and decreased Gata-3 level in OVA-sensitized mice. This indicates that P2X4R antagonist, 5-BDBD, could ameliorate airway Th2 inflammation in allergic asthma in mice, which were likely mediated, at least partly, by altering the Th1/Th2 via re-balancing the related transcription factor, T-bet/Gata-3. These results showed that P2X4R, as well as other P2XRs, could act on allergic inflammation by modulating the T cell response, but maybe they play their roles by different signaling pathways or by pathways crosstalk.

It is believed that the activation of phosphate-p38 MAPK can affect Th2 cytokines secretion by regulating expression of transcription factors (32, 33). Thus, we also studied the effect of 5-BDBD on the activation of p38 MAPK. Thl/Th2 imbalance disorders, especially Th2 cell hyperactivity, play the key roles in airway inflammation in allergic asthma. Moreover, transcription factor T-bet and GATA-3 could induce Th1/Th2 polarity and secretion of the effector cytokines. Here, the results imply that 5-BDBD may inhibit allergic inflammation by alleviating Thl/Th2 imbalance disorders. Moreover, they hint that P2X4R-p38 MAPK signaling might be involved in the pathological process.

## Conclusion

The current study suggests that P2X4R antagonist, 5-BDBD may inhibit allergic inflammation by the reduction of Th2 cytokines production in ovalbumin-sensitized mice. The process may involve the p38 MAPK pathway by altering Th1/Th2 via re-balancing the related transcription factor, T-bet/Gata-3. But it is unclear whether there is a crosstalk with another intracellular signaling. Therefore, further study is still needed to clarify this novel signaling pathway.

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## Conflicts of interest

The authors declare that there is no conflicts of interest in this study.

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