

# Apyrase protects against allergic airway inflammation by decreasing the chemotactic migration of dendritic cells in mice

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Received January 21, 2014; Accepted April 25, 2014

DOI: 10.3892/ijmm.2014.1771

**Abstract.** Recent studies have demonstrated that extracellular adenosine 5'-triphosphate (eATP) is involved in allergic airway inflammation by activating purinergic receptors. eATP can be hydrolyzed by ectonucleotidases, such as CD39. In this study, we investigated the expression and distribution of CD39 in the lungs of mice, as well as the effects of apyrase on airway inflammation and the chemotactic migration of dendritic cells (DCs). A mouse model of asthma was developed with chicken ovalbumin (OVA)/aluminum hydroxide using female C57BL/6 mice. Apyrase was administered to OVA-sensitized mice prior to each challenge by intraperitoneal injection. The distribution of CD39 was detected by immunofluorescence. The mRNA and protein expression of CD39 was determined by quantitative PCR and western blot analysis, respectively. The levels of Th2 cytokines in the bronchoalveolar lavage fluid (BALF) were measured by enzyme-linked immunosorbent assay (ELISA). The effect of apyrase on the chemotactic migration of DCs towards ATP was explored by migration assay *in vitro*. In the lungs, CD39 was primarily located in the cytoplasm and cytomembrane of bronchial epithelial cells and CD39 expression was reduced in mice with allergic asthma. Treatment with apyrase markedly attenuated OVA-induced airway inflammation, including peribronchial eosinophilic inflammation and reduced the number of inflammatory cells, as well as the levels of cytokines in BALF. Furthermore, apyrase also markedly reduced the expression of GATA binding protein 3 (GATA3) and decreased the chemotactic migration of DCs towards ATP. Our data demonstrate that a reduction in CD39 expression may be associated with the development of allergic airway inflammation and that apyrase alleviates airway inflammation by decreasing the chemotactic migration of DCs towards eATP. Therefore, targeting at eATP or ectonucleotidases may provide a novel therapeutic approach for allergic asthma.

## Introduction

Asthma is a chronic inflammatory disorder of the airways and is characterized by airway inflammation, airway hyperresponsiveness (AHR) to non-specific stimuli, as well as airway remodeling. Th2 cells are classically thought to drive the development of asthma through the release of Th2 cytokines, which are indispensable for the synthesis of immunoglobulin E (IgE), mucus production and AHR (1-3). In addition to Th2 cell-mediated adaptive immune response, asthma is strongly influenced by the innate immune responses from airway cells, such as epithelial cells, mast cells, natural killer T cells and dendritic cells (DCs) (4,5).

Adenosine 5'-triphosphate (ATP), as an endogenous danger signal, has unique features. It is found at high concentrations in the intracellular cytoplasm, at low levels in the extracellular or pericellular space in healthy tissue, is rapidly released upon cell damage and is inactivated by the powerful ubiquitous ectonucleoside triphosphate diphosphohydrolase (E-NTPDase) (6,7). Extracellular ATP (eATP) and other nucleotides [e.g., adenosine diphosphate (ADP), uridine 5'-triphosphate (UTP) and uridine diphosphate (UDP)] exert their effects by binding to purinergic P2-receptors (P2R), which can be subdivided into 2 families: the G-protein-coupled P2YR and the ligand-gated ion channel, P2XR. Both P2XR and P2YR are present in the lungs. Recent studies have indicated that eATP contributes to the pathogenesis of asthma through purinergic receptors, such as P2Y<sub>2</sub>, P2Y<sub>4</sub>, P2Y<sub>6</sub> and P2X<sub>7</sub> receptors (8-11).

However, purinergic signaling pathways are regulated by CD39/E-NTPDase1. CD39 belongs to the E-NTPDase family and is also known as E-NTPDase1. CD39 is primarily expressed on vascular endothelial cells and immune cells and hydrolyzes extracellular ATP and ADP to adenosine monophosphate (AMP). As ATP is typically a pro-inflammatory mediator and AMP is rapidly converted to the anti-inflammatory metabolite, adenosine, by ecto-5'-nucleotidase (also known as CD73), CD39 tends to promote an anti-inflammatory and immune suppressive milieu (7,12). Compelling studies have demonstrated the important roles of CD39 in the immunoregulatory function (13-17).

Given that extracellular nucleotides play important roles in the pathogenesis of asthma, little is known about the role of associated ectonucleotidases, such as CD39 in allergic asthma. Thereby, we hypothesized that CD39 expression is abnormal in allergic asthma and that the exogenous supplementation of apyrase may attenuate allergic airway inflammation. To

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**Key words:** allergic asthma, CD39, apyrase, adenosine 5'-triphosphate, dendritic cells

confirm these assumptions, we first analyzed CD39 expression levels in the lungs of asthmatic mice. Second, airway inflammation was evaluated in ovalbumin (OVA)-sensitized mice treated with apyrase prior to each OVA challenge. Third, the expression of GATA binding protein (GATA3), which controls Th2 cell differentiation, was measured. Finally, we attempted to explore whether apyrase affects the chemotactic migration of DCs towards eATP.

## Materials and methods

**Animals.** Female 6- to 8-week old C57BL/6 mice were obtained from the Center for Animal Experiment of Wuhan University (Wuhan, China) and maintained in the Animal Biosafety Level 3 Laboratory of the university. All animals were bred at the animal facilities under specific pathogen-free conditions. Animal experiments were conducted under the approval of the Institutional Animal Care and Use Committee of Wuhan University.

**Murine model of allergic asthma.** The mice were immunized intraperitoneally with 20  $\mu$ g OVA (Sigma-Aldrich, St. Louis, MO, USA) adsorbed to 2 mg aluminum hydroxide (Thermo Fisher Scientific Inc., Rockford, IL, USA) in 200  $\mu$ l PBS on days 0 and 14 and challenged with 100  $\mu$ g OVA in 50  $\mu$ l PBS by intranasal (i.n.) administration for over 3 consecutive days (days 25-27). Age- and gender-matched control mice were treated in the same manner with PBS as a substitute for OVA. Apyrase (diluted with PBS, 0.2 IU/g body weight; Sigma-Aldrich) or PBS was intraperitoneally administered to the OVA-sensitized mice prior to each OVA challenge.

**Histological analysis and immunofluorescence.** At 48 h after the final challenge, the mice were sacrificed and the left lungs were excised. The lungs were fixed in 4% paraformaldehyde (PFA) buffer, dehydrated, embedded and sectioned. Paraffin-embedded 5- $\mu$ m sections were stained with hematoxylin and eosin (H&E) to evaluate airway inflammation and periodic acid-Schiff (PAS) to evaluate goblet cell hyperplasia and mucus secretion. A total of 4-5 sections were evaluated per lung under a magnification of x200. The inflammation degree of peribronchial and perivascular regions was assessed on a subjective scale of 0-3, as previously described (18). A value of 0 was regarded as undetectable inflammation, a value of 1 as occasional cuffing with inflammatory cells, a value of 2 as bronchi or vessels surrounded by a thin layer of inflammatory cells (1-5 layer cells), and a value of 3 was regarded as bronchi or vessels surrounded by a thick layer of inflammatory cells (>5 layers of cells).

The location of CD39 in the lungs obtained from the mice was detected by immunofluorescence. Briefly, 5- $\mu$ m-thick sections were incubated with a rabbit anti-mouse CD39 antibody (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) overnight at 4°C. For detection, Rhodamine-labeled goat anti-rabbit secondary antibody (Santa Cruz Biotechnology, Inc.) was used. The nuclei were stained with 4,6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI; Sigma-Aldrich).

**Collection of cells and supernatants in the bronchoalveolar lavage fluid (BALF).** At 48 h after the final challenge, the mice

Table I. Primer sequences and product sizes.

Gene name	Primer sequences (5'→3')	Product size (bp)
CD39/ENTPD-1		
Sense	CATCCAAGCATCACCAGACT	154
Antisense	ATGAT CTTGGCACCCTGGAA	
GATA3		
Sense	AGGGACATCCTGCGCGAACTGT	166
Antisense	CATCTTCCGGTTTCGGGTCTGG	
GAPDH		
Sense	TGTGTCCGTCGTGGATCTGA	150
Antisense	TTGCTGTTGAAGTCGCAGGAG	

CD39/ENTPD-1, ectonucleoside triphosphate diphosphohydrolase-1; GATA3, GATA binding protein 3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

were sacrificed and bronchoalveolar lavage was performed 3 times with 0.5-ml aliquots of PBS containing 1 mM sodium EDTA. Cells in the BALF were collected by centrifugation and were counted with a hemocytometer. Smears of cells were prepared by cytospin and stained with Wright-Giemsa in order to differentiate the inflammatory cells (eosinophils, neutrophils and lymphocytes). Approximately 400 cells were counted in each random location. The supernatants in the BALF were collected and stored at -70°C until use.

**RNA extraction and quantitative PCR (qPCR).** The right upper lung lobes were measured for gene transcript levels. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and cDNA was synthesized using ReverTra Ace qPCR RT Master Mix (Toyobo, Tokyo, Japan) according to the manufacturer's instructions. qPCR was conducted in triplicate using SYBR Premix Ex Taq™ (Takara Bio, Inc., Otsu, Japan). The primer sequences are presented in Table I. The thermal cycling conditions were as follows: denaturation at 95°C for 1 min, followed by 40 cycles of denaturation at 95°C for 10 sec, annealing at 61°C for 10 sec and extension at 72°C for 10 sec. The data were normalized to the internal reference gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The relative expression of the target gene was calculated using the comparative  $2^{-\Delta\Delta C_t}$  method.

**Western blot analysis.** Protein was extracted from the right median lobes using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime Institute of Biotechnology, Haimen, China). Total protein (60  $\mu$ g) was separated by electrophoresis on SDS-PAGE gels, transferred onto PVDF membranes (Millipore Corp., Billerica, MA, USA), blocked with TBST-containing 5% non-fat dried milk at room temperature for 1 h and probed with rabbit anti-mouse CD39 or GAPDH (1:200, Santa Cruz Biotechnology, Inc.). The membranes were incubated with primary antibody overnight at 4°C and then with horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (1:5,000; Santa Cruz Biotechnology, Inc.) at room temperature for 1 h. Chemoluminescence images were

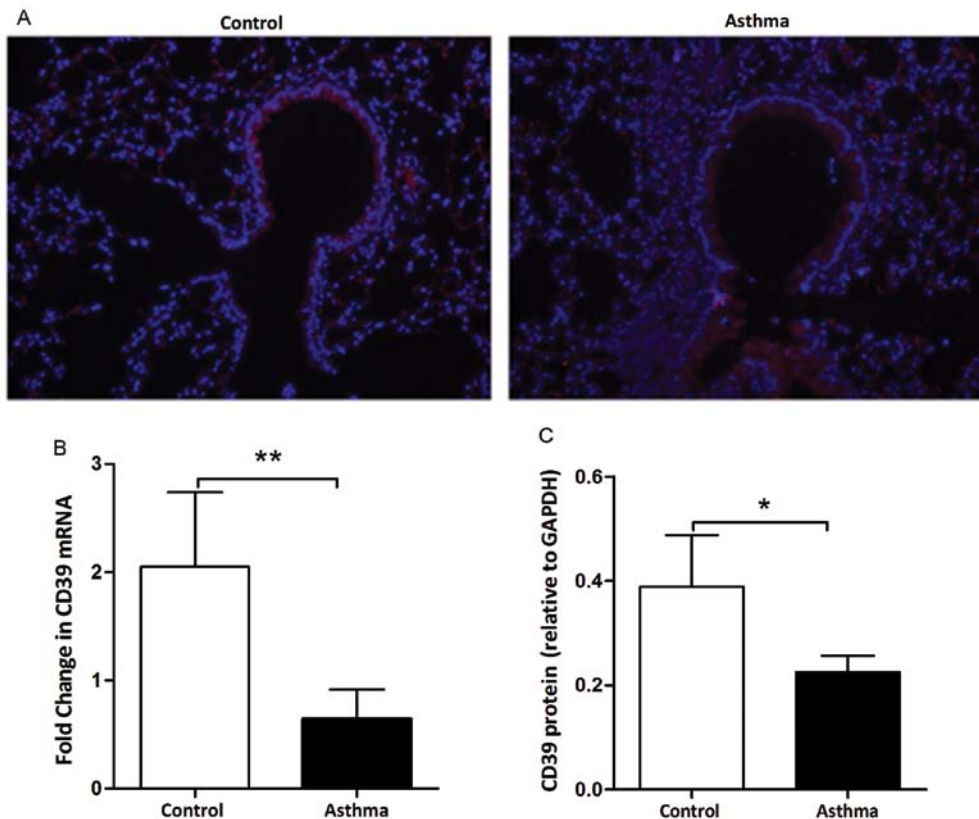


Figure 1. Ovalbumin (OVA)-induced airway inflammation leads to a reduction in CD39 expression. (A) Representative micrographs of immunofluorescence of CD39 in the lungs from the control and asthmatic (asthma) mice are shown. CD39 was detected with Rhodamine-labeled secondary antibody and located at the cytomembrane and cytoplasm of bronchial epithelial cells. Control: PBS sensitization and challenge; asthma, OVA sensitization and challenge. (B) CD39 mRNA expression was significantly reduced in the lungs from asthmatic mice compared to control mice. (C) CD39 protein expression was significantly reduced in the lungs from asthma mice compared to control mice. One representative experiment of 3 is shown. The data are presented as the means  $\pm$  SD, (n=4-6 mice per group). \*\*P<0.01 and \*P<0.05; original magnification, x200.

captured with ECL (Beyotime Institute of Biotechnology) using the Fusion Fx7 image acquisition system (Vilber Lourmat, Marne La Vallée, France). The quantification of the bands was performed by densitometry using ImageJ software. The CD39 protein expression level was calculated by densitometry relative to GAPDH.

**Detection of cytokines.** The levels of cytokines were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions. The lower limit of detection was 4 pg/ml for interleukin (IL)-4 and IL-5. All ELISA kits for cytokines were purchased from eBioscience, Inc. (San Diego, CA, USA).

**Generation of bone marrow-derived DCs (BMDCs).** Immature DCs were prepared from bone marrow progenitors. Bone marrow mononuclear cells were prepared from femur bone marrow suspensions of C57BL/6 male mice (5-6 weeks old) and then cultured at a density of  $2 \times 10^6$  cells/ml in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 10 ng/ml recombinant murine granulocyte-macrophage colony-stimulating factor (GM-CSF) and 10 ng/ml recombinant murine IL-4 (both from PeproTech, Rocky Hill, NJ, USA). Non-adherent cells were gently washed out on the 3rd day of culture. On days 5 and 7, the medium was refreshed. The purity of the DCs was at least 90%. On day 8, the BMDCs were incubated with

1  $\mu$ g/ml lipopolysaccharide (LPS; Sigma-Aldrich) overnight and collected for subsequent experiments.

**Migration assay of DCs in vitro.** A migration assay was performed in 24-well Transwell chambers with 5  $\mu$ m pore size polycarbonate filters (Corning Life Sciences, Tewksbury, MA, USA). Various concentrations of ATP diluted in RPMI-1640 containing 0.5% bovine serum albumin (BSA) were added to the lower chambers in a volume of 0.6 ml; 0.1 ml RPMI-1640 containing DCs ( $2 \times 10^5$  cells/well) was added to the upper chambers followed by culture at 37°C for 4 h. In some experiments, apyrase (1 IU) was added to the medium containing ATP in the lower chambers. The number of migrated DCs to the lower chambers was calculated using a hemocytometer. The results are presented as the chemotactic index and calculated as the number of cells in the lower chamber containing the different stimuli divided by the number of cells in the chamber containing medium alone.

**Statistical analysis.** The data are expressed as the means  $\pm$  SD, representing at least 2 independent experiments with consistent results (n=4-6 mice per group). The data from 1 representative experiment of 3 are shown. The differences were compared using the unpaired Student's t-test for 2 groups or one-way ANOVA analysis for 3 groups. Statistical analysis was performed using SPSS 17.0 software (IBM SPSS, Chicago, IL,

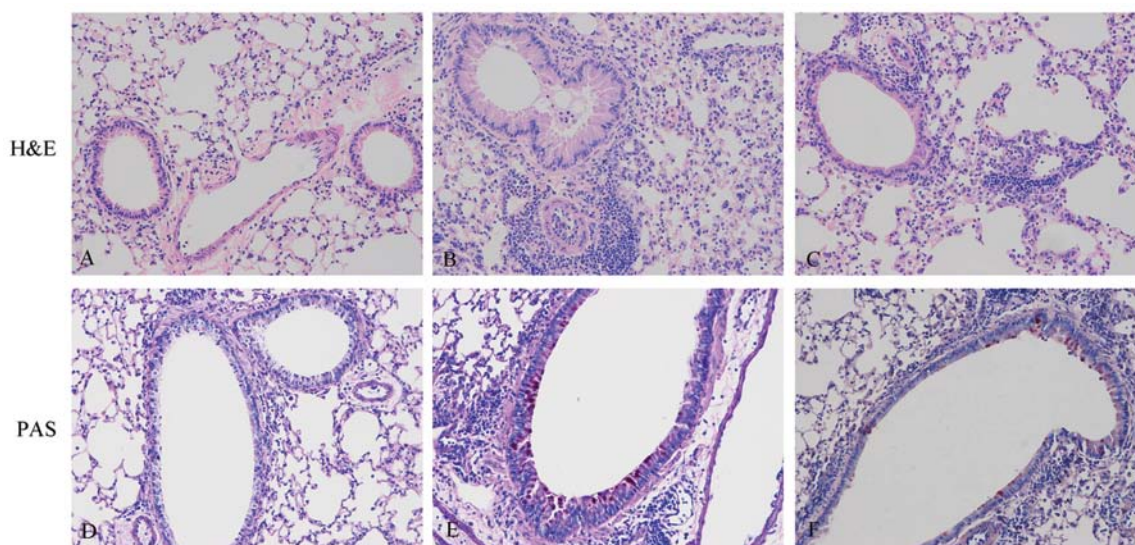


Figure 2. Apyrase attenuates ovalbumin (OVA)-induced tissue eosinophilia and mucus production. (A-C) Representative micrographs of hematoxylin and eosin (H&E) staining of lung sections are shown. (D-F) Representative micrographs of periodic acid-Schiff (PAS) staining of lung sections are shown. The group was coded as sensitization/treatment/challenge. (A and D) PBS/PBS/PBS. (B and E) OVA/PBS/OVA. (C and F) OVA/apyrase/OVA. (n=4-6 mice per group).

USA). A value of  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

*OVA-induced airway inflammation leads to a reduced expression of CD39 in lung tissue.* We investigated the distribution of CD39 in the lungs obtained from mice by immunofluorescence assay. As shown in Fig. 1A, CD39 was located at the cytomembrane and cytoplasm of bronchial epithelial cells (red area indicates the positive location). The fluorescence intensity was reduced in the lungs from OVA-sensitized and challenged (asthma) mice compared to that in the lungs from PBS-sensitized and challenged (control) mice. We further assessed the expression levels of CD39 in the lungs from the control and asthmatic mice. At the mRNA level, CD39 expression was decreased in the asthmatic mice, as shown by qPCR (Fig. 1B). A marked decrease in CD39 protein expression was also observed in the asthmatic mice, as shown by western blot analysis (Fig. 1C). These data indicate that a reduction in CD39 expression is associated with OVA-induced allergic airway inflammation.

*Treatment with apyrase attenuates OVA-induced tissue eosinophilia, mucus production and airway inflammation.* Having shown that a reduced CD39 expression is associated with OVA-induced allergic airway inflammation, we exogenously supplemented apyrase, an eATP scavenger, to investigate its effects on airway inflammation. Lung tissues were collected 48 h after the final OVA challenge. In histological analysis, the OVA-exposed mice showed cardinal pathological features of asthma-like inflammation. In contrast to the PBS-sensitized and challenged controls (Fig. 2A), OVA-sensitized and challenged mice displayed numerous inflammatory cells in the peribronchovascular and perivascular zones (Fig. 2B). Compared to treatment with PBS, treatment with apyrase markedly decreased the number of eosinophil-rich inflammatory cells infiltrating the peribronchovascular and perivascular regions (Fig. 2C). As shown in Fig. 2D-F,

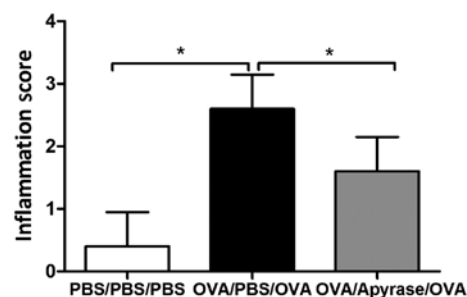


Figure 3. Apyrase reduces the inflammation score in ovalbumin (OVA)-exposed mice. Peribronchial and perivascular inflammation was measured 48 h after the final challenge. The inflammation score was significantly increased in the OVA-sensitized and challenged mice and the administration of apyrase decreased the inflammation score. The group was coded as sensitization/treatment/challenge. The data are presented as the means  $\pm$  SD. (n=4-6 mice per group). \* $P < 0.05$ .

PAS staining was used to detect goblet cells. In contrast to the controls (Fig. 2D), the OVA-exposed mice (Fig. 2E) showed goblet cell hyperplasia in the airways, which was significantly reduced by treatment with apyrase (Fig. 2F). OVA exposure markedly increased the inflammation scores of the peribronchial and perivascular regions (Fig. 3), compared to PBS sensitization and challenge. The increased lung inflammation following exposure to OVA was reduced by approximately 40% following treatment with apyrase. Taken together, these results reveal that apyrase significantly reduces OVA-induced inflammatory cell infiltration into the lungs, goblet cell hyperplasia in the airways and airway inflammation.

*Treatment with apyrase reduces the number of inflammatory cells in BALF following exposure to OVA.* BALF was collected 48 h after the final OVA challenge and the number of the total cells and the different inflammatory cells were counted. Exposure to OVA markedly increased the number of eosinophils, lymphocytes and neutrophils, compared to PBS

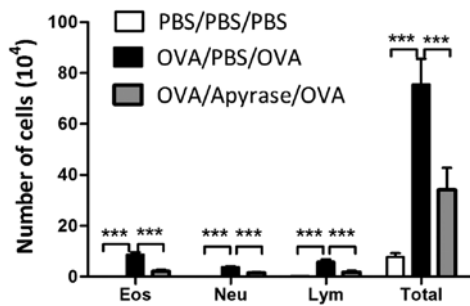


Figure 4. Apyrase reduces ovalbumin (OVA)-induced inflammatory cells in bronchoalveolar lavage fluid (BALF). Differential cell counts were performed on approximately 400 cells in each of 4 different random locations to identify eosinophils (Eos), lymphocytes (Lym) and neutrophils (Neu). The group was coded as sensitization/treatment/challenge. The data are presented as the means  $\pm$  SD, (n=4-6 mice per group). \*\*\*P<0.001.

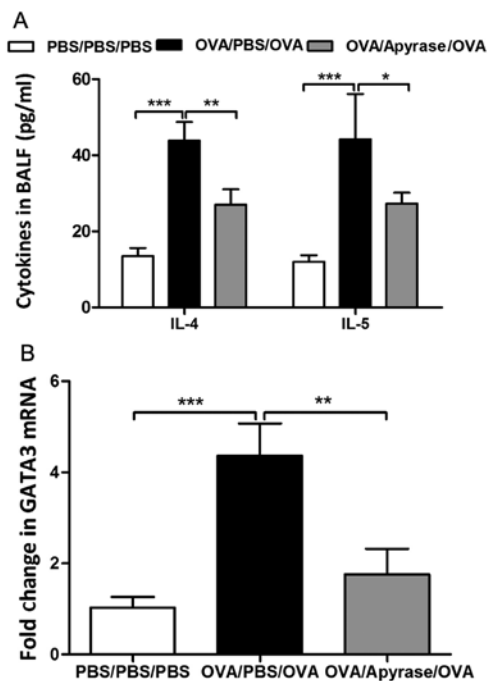


Figure 5. The levels of Th2 cytokines in the bronchoalveolar lavage fluid (BALF) and the expression of GATA3 in the lungs were significantly decreased in the apyrase-treated mice. (A) The levels of cytokines in the BALF were increased in the OVA-exposed mice and the increased levels were decreased by apyrase. (B) The expression of GATA3 in the lung tissues was increased in the ovalbumin (OVA)-exposed mice and the increased level was decreased by apyrase. The group was coded as sensitization/treatment/challenge. The data are presented as the means  $\pm$  SD. (n=4-6 mice per group). \*\*\*P<0.001, \*\*P<0.01, \*P<0.05.

sensitization and challenge (Fig. 4). The administration of apyrase markedly reduced the number of eosinophils, lymphocytes and neutrophils in BALF, compared to the PBS-treated asthmatic mice. In addition, a reduction in the number of total cells was observed in the apyrase-treated mice.

*Administration of apyrase markedly decreases the levels of Th2 cytokines in BALF and the expression of GATA3 in lung tissue.* Given the essential role of Th2 cytokines in triggering the allergic inflammatory response, we detected the levels of IL-4 and IL-5 in BALF. OVA sensitization and challenge

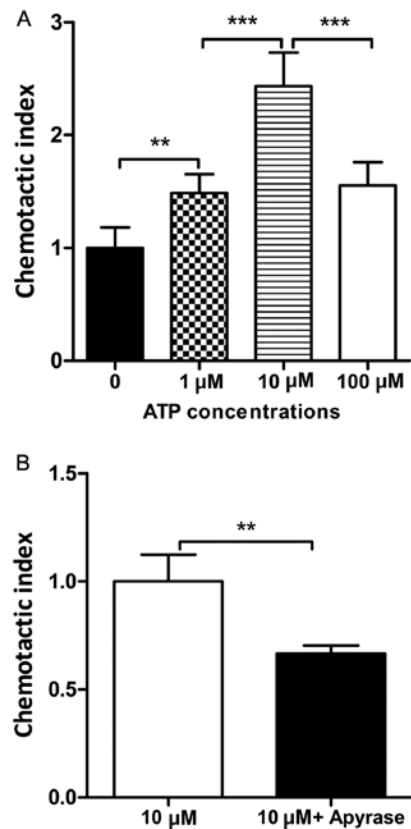


Figure 6. Apyrase decreased the chemotactic migration of dendritic cells (DCs) towards adenosine 5'-triphosphate (ATP). (A) A two-chamber Transwell system was used to evaluate the chemotactic migration of DCs towards ATP (various concentrations) in an *in vitro* assay. (B) Apyrase decreased the chemotactic migration of DCs towards ATP. The data are presented as the means  $\pm$  SD. \*\*P<0.01, \*\*\*P<0.001. One representative experiment of 3 is shown.

markedly increased the concentration of cytokines in BALF, as shown by ELISA (Fig. 5A) compared to PBS sensitization and challenge. The increased levels of cytokines in BALF were significantly reduced by apyrase treatment.

GATA3, the key transcription factor, controls the differentiation of Th2 cells. We further determined whether apyrase decreases GATA3 mRNA expression in the lungs obtained from asthmatic mice. In the OVA-exposed mice, the mRNA level of GATA3 was increased by 4-fold in the lung tissues compared with that in the PBS-sensitized and challenged control mice (Fig. 5B). The administration of apyrase effectively reduced the expression of GATA3 in the lung tissues compared with the PBS-treated asthmatic mice (Fig. 5B).

*Apyrase decreases the chemotactic migration of DCs towards ATP.* Our results revealed that apyrase attenuated airway inflammation. ATP is a chemoattractant for DCs *in vitro* (8,19). Therefore, we explored whether apyrase decreases the chemotactic migration of DCs towards ATP. Various concentrations of ATP were added to the medium in the lower chamber. As shown in Fig. 6A, the DCs showed the strongest chemotactic activity with 10  $\mu$ M ATP. Apyrase was then added to the medium containing 10  $\mu$ M ATP. We found that upon the administration of apyrase, the chemotactic migration of DCs towards ATP was markedly decreased compared to the cells treated with ATP alone (Fig. 6B).

## Discussion

CD39 is an integral membrane protein that hydrolyzes ATP and is constitutively expressed in the spleen, thymus, lung and placenta (20,21). CD39 is structurally characterized by 2 trans-membrane regions, a small cytoplasmic region and a large extracellular region that is essential for the catabolic activity of the enzyme (22). Increasing evidence demonstrates the regulation of immunity by CD39 in infectious diseases (23-26), autoimmune diseases (27-29) and ischemia-reperfusion injury (30,31). However, little is known of the role of CD39 in allergic asthma.

In the present study, we found that CD39 was located at the surface of bronchial epithelial cells and the CD39 expression was reduced in the lungs obtained from allergic asthmatic mice, which was associated with the pathogenesis of allergic asthma. In addition, CD39 expression is decreased in patients with Crohn's disease (32). Furthermore, the deletion of CD39 has been demonstrated to increase the disease severity and pre-treatment of apyrase has been shown to improve the outcome in a murine model of DSS-induced colitis (32). These findings demonstrate a protective role of CD39 in inflammatory diseases. In our study, the administration of apyrase, a soluble factor with enzymatic activity essentially identical to CD39, to OVA-sensitized mice attenuated airway inflammation, which was consistent with the results of a previous study (8). This mainly included a significant decrease in airway eosinophilic inflammation, goblet cell hyperplasia and the levels of Th2 cytokines in BALF.

Th2 cells play a crucial role in allergic asthma by triggering the recruitment of eosinophils and mast cells and inducing goblet cell hyperplasia (33). However, the polarization of Th2 cells is regulated by the transcription factor, GATA3 (34). In our study, the expression of GATA3 was markedly increased in the lungs obtained from OVA-sensitized and challenged mice compared to those from PBS-sensitized and challenged mice. In a previous study of ours, GATA3 mRNA expression was decreased in allergic asthma patients (33). Treatment with apyrase reduced its expression in the lungs, which resulted in the reduced levels of Th2-cytokines.

DCs are pivotal for the initiation and maintenance of adaptive Th2 cell responses to inhaled allergens in asthma (4). Our findings demonstrated that ATP induced the migration of DCs, which was in accordance with a previous study that demonstrated that ATP promoted the migration of DCs by activating P2Y<sub>2</sub> receptor (9). However, in our study, apyrase reduced the chemotactic migration of DCs, which was associated with the hydrolysis of ATP by apyrase. Furthermore, in another study of ours, we showed that ATP induced the migration of mast cells and the exogenous administration of soluble CD39 inhibited the migration of ATP-induced mast cells in an *in vitro* assay (unpublished data).

In conclusion, the present study provides evidence of the role of associated ectonucleotidases in a murine model of OVA/aluminum hydroxide-induced allergic asthma. As shown by our results, the reduced CD39 expression was associated with the development of allergic airway inflammation. Apyrase decreased the expression of GATA3 and reduced the chemotactic migration of DCs towards ATP, which resulted in the alleviation of airway inflammation. Thereby, targeting

eATP or ectonucleotidases may provide a novel therapeutic approach for allergic asthma.

## Acknowledgements

We would like to thank the National Natural Science Foundation of China. We would also like to thank the faculties at the Center for Medical Research, Hubei Key Laboratory of Allergy and Immune-Related Disease of Wuhan University. This study was supported by a grant from the National Natural Science Foundation of China (81170029).

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