Am J Transl Res 2020;12(7):4074-4083
www.ajtr.org /ISSN:1943-8141/AJTR0109137

Original Article

Blocking pannexin1 reduces airway inflammation in a murine model of asthma

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Received February 13, 2020; Accepted May 31, 2020; Epub July 15, 2020; Published July 30, 2020

Abstract: Stressed or injured cells release ATP into the extracellular milieu via the pannexin1 (Panx1) channels, which is the basis of inflammation in a variety of conditions, including allergic lung inflammation. Although the role of Panx1 in mediating inflammation has been well established, the role of its mimetic peptide, ^{10}Panx1, which inhibits ATP release from Panx1 channels, in allergic asthma remains understudied. The aim of this study was to evaluate the effects of using ^{10}Panx1 to inhibit Panx1 channel in a murine model of ovalbumin (OVA)-induced asthma. We demonstrate that blockade of Panx1 significantly attenuated goblet cell hyperplasia and inflammatory cell infiltration into the lungs of OVA-sensitized mice. Inhibition of Panx1 also reduced the total and eosinophil cell numbers in the bronchoalveolar lavage fluid (BALF) and reduced expression of CCL11 and CCL2 in lung tissues from mice. Moreover, we detected lower levels of IL-5 and IL-13 in the culture supernatant of OVA-restimulated splenocytes from ^{10}Panx1-treated mice. Collectively, our findings suggest that Panx1 inhibition of allergen-mediated lung inflammation has the potential to suppress allergic responses in asthma.

Keywords: EXtracellular ATP, pannexin1, ^{10}Panx1, chemokine, lung inflammation, asthma

Introduction

Asthma is a complex, chronic inflammatory disease of the airways that affects about approximately 300 million people worldwide. It is characterized by airway inflammation, reversible airway obstruction, airway hyperreactivity, and airway remodeling, which are facilitated by the synergistic interaction of the innate and adaptive immune cells with airway epithelial cells [1, 2]. Cytokines of T-helper type 2 (Th2) cells and chemokines of innate immune cells, including CCL2 and CCL11, work together in the pathophysiological process of asthma and become the focus of targeted asthma therapy [2, 3].

Although Th2 immunity plays a crucial role in the pathophysiology of asthma, there is increasing evidence that airway epithelial cells release danger signals (such as ATP, HMGB1, and uric acid) against inhaled allergen that can promote the induction of allergic responses [4]. Among the danger signals, ATP has been identified as a mediator of lung inflammation in asthma and has been shown to accumulate in the airways of asthmatic patients and mice [5, 6]. ATP is released into the extracellular space by stressed, injured, or apoptotic cells during tissue stress or damage, causing the recruitment of inflammatory cells to the inflammatory sites by binding to purinergic receptors [7, 8].

Various mechanisms mediate the extracellular release of ATP; however, the pannexin (Panx) channels are important for ATP release [9]. The Panx channels are non-junctional plasma membrane hemichannels whose opening is induced by various mechanisms during innate and adap-

tive immune responses [10]. In vertebrates, the Panx family comprises three subtypes, Panx1, 2, and 3 [9, 10]. Among the subtypes, Panx1 is the most ubiquitously expressed and is present in a variety of tissues and cell types, including the airway epithelium and cells of the immune system [10, 11]. Panx1-mediated ATP release can drive inflammation through activation of T cells, production of proinflammatory cytokines, and the activation and chemotaxis of innate immune cells [12, 13]. Panx1 has been implicated in the pathogenesis of diseases such as inflammatory bowel diseases, pulmonary fibrosis, and chronic obstructive pulmonary disease [9-13]. ATP release from the Panx1 channels can be inhibited using the mimetic peptide, ^{10}Panx1, which mimics the loop-to-loop interaction between two hemichannels [9, 11].

Multiple lines of evidence suggest that targeting Panx1 may be beneficial in the treatment of inflammatory diseases [14-16]. Thus, Panx1 may be a target for alleviating the inflammatory responses in asthma. We investigated the therapeutic potential of targeting Panx1 in a murine model of ovalbumin (OVA)-induced asthma using the Panx1-mimetic peptide ^{10}Panx1.

Materials and methods

Animals

Female BALB/c mice were purchased from the National Laboratory Animal Center, Taiwan. The mice were housed and maintained by the animal care facility of Chang Gung University. The housing, care, and experimental procedure were carried out in accordance with the guidelines of Chang Gung University Institutional Animal Care and Use Committee (IACUC Approval number 106-029).

Allergen sensitization, challenge, and drug treatment

Mice were sensitized intraperitoneally (I.P.) on days 0 and 7 with 100 μg OVA (Sigma-Aldrich, St, Louis, MO, USA) together with 2 mg aluminum hydroxide (Alum, Thermo Fisher Scientific, Rockford, IL, USA) in 200 μl normal saline. On days 19, 22, 26, 29, and 33, the mice were challenged by intratracheally (I.T.) with 10 μg OVA. For peptide treatments, 1.5 mg/kg and 3 mg/kg of ^{10}Panx1 (synthesized by Kelowna Int'l Scientific, New Taipei City, Taiwan) or 3 mg/kg of scrambled (SCR) peptide (Kelowna Int'l Scientific) were I.T. administered to mice together with the 10 μg OVA in 50 μl of PBS on days 19, 22, 26, 29, and 33. On day 34, mice were sacrificed to analyze cell infiltration, ATP levels, and cytokine, or chemokine production.

Histological analysis

At sacrifice, the lungs were collected without lavage and fixed with 4% formaldehyde, embedded in paraffin and sectioned. Lung sections were stained with hematoxylin and eosin (H&E) for lung eosinophilia analysis or with periodic acid-Schiff (PAS) for goblet cell hyperplasia and mucus evaluation. Digital images were obtained at 200 × magnification using an Olympus 1X71 microscope with DP controller software (Olympus America Inc, Melville, NY, USA) and the quantitation of digital images was performed by MetaMorph software (Molecular Devices, San Jose, CA, USA).

Collection of bronchoalveolar lavage fluid and counting of cells

Twenty-four hours after the final OVA challenged, mice were sacrificed, lungs were lavaged with 1.5 ml PBS, bronchoalveolar lavage fluid (BALF) was collected, and cell counts were performed using a hemocytometer. Cells were also smeared on frosted slides using Cytospin 4 (Thermo Fisher Scientific, Pittsburg, PA, USA) and stained with Wright-Giemsa stain for differential cell counting. Two hundred cells were examined for each sample.

Extracellular ATP measurement

To detect extracellular ATP levels, the lungs of mice were lavaged with PBS containing 5 mM EDTA to minimize catabolism of ATP. The BALF samples were supplemented with 5 mM $MgCl_2$, and ATP levels were measured with an ATPlite detection kit (PerkinElmer, Waltham, MA, USA) and luminescence was detected using a luminometer (GloMax, Promega, Madison, WI, USA). The concentration of ATP in each BALF sample was determined using a known concentration of the ATP standard according to the manufacturer's instructions.

In vitro OVA restimulation of splenocytes

Splenocytes were isolated from all groups of mice. Suspended single splenocytes were seeded in a 24 well plate at a concentration of

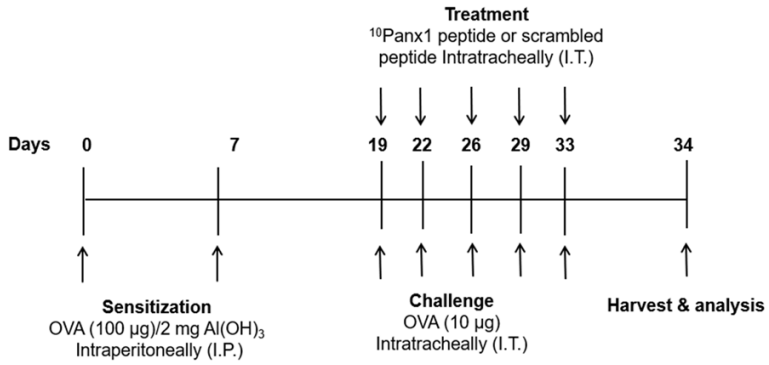

Figure 1. Sensitization model of ovalbumin (OVA)-induced allergic airway inflammation. This diagram depicts the OVA-induced airway inflammation model used in the study. On days 0 and 7, mice were sensitized by intraperitoneal injection (I.P.) with 100 μg OVA with 2 mg Alum in 200 μl normal saline. On days 19, 22, 26, 29, and 33, mice were challenged with 10 μg of OVA intratracheally (I.T.) together with 1.5 or 3 mg/kg ^{10}Panx1 for the treatment groups and 3 mg/kg of scrambled Panx1 peptide for the control group. Twenty four hours after the last challenge, mice were sacrificed and analyzed.

5×10^6 cells/ml in RPMI 1640 medium and stimulated with 100 μg/ml OVA. The stimulation lasted for 6 days, and the levels of Th2-related cytokines in the supernatants were measured by ELISA.

Detection of antibodies, chemokines, and cytokines

The levels of OVA-specific IgG1 and IgE, CCL2, CCL11, IL-4, IL-5, and IL-13 were assayed by ELISA according to the manufacture's instruction. The ELISA kits for OVA-specific antibodies and IL-5 were purchased from BD Biosciences (San Jose, CA, USA) and ELISA kits for CCL2, CCL11, IL-4, and IL-13 were purchased from R&D Systems (Minneapolis, MN, USA).

Gene expression analysis

RNA from lung tissues was isolated using TRIzol reagent (Carlsbad, CA, USA). The MMLV Reverse Transcription Kit (PROTECH, Taipei, Taiwan) was used to synthesize cDNA. The expression of CCL2 (5'-TTAAAAACCTGGATCGGAACCAA, 3'-GCATTAGCTTCAGATTTACGGGT) and CCL11 (5'-GAATCACCAACAACAGATGCAC, 3'-ATCCTGGACCCACTTCTTCTT) were measured with iQ™ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). Primers were synthesized by Tri-biotech (Taipei, Taiwan).

Statistical analysis

All data are presented as mean ± SEM and analyzed using Prism software (GraphPad Software, La Jolla, CA, USA). Statistical significance between groups was calculated by the Kruskal-Wallis test and then by uncorrected Dunn's test. A *P*-value of < 0.05 was considered significant between groups and expressed as *P < 0.05, **P < 0.01, ***P < 0.001.

Results

Blocking Panx1 suppresses airway inflammation

To investigate whether Panx1 blockade treatment alleviates airway inflammation, we used a murine model of OVA-induced asthma (**Figure 1**). Twenty-four hours after the last OVA challenge, lung tissues were collected to assess airway inflammation. Hematoxylin and eosin (H&E)-stained lung sections were evaluated. Histological analysis of lung sections revealed increased accumulation of eosinophils in the peribronchial region of OVA-sensitized mice compared with healthy controls. The administration of 3 mg/kg of ^{10}Panx1 (P3 group) reduced the infiltration of eosinophils in the peribronchial region (**Figure 2A** top panel and **2B**). We further evaluated goblet cell hyperplasia and mucus secretion using periodic acid-Schiff (PAS) staining. Mice in the P3 group had significantly less goblet cell hyperplasia, and mucus secretion compared to OVA-sensitized and scrambled peptide-treated (SCR) control groups (**Figure 2A** bottom panel and **2C**).

Inhibiting Panx1 reduces the number of cells and ATP levels in the bronchoalveolar lavage fluid of mice

Next, we examined whether Panx1 blockade affects eosinophilic infiltration and extracellular ATP release in BALF. Total and differential population cell numbers were counted. The total number of infiltrating cells in BALF was reduced in mice treated with 3 mg/kg of ^{10}Panx1 compared to OVA-sensitized mice (**Figure 3A**). Blocking Panx1 also reduced the number (**Figure 3B**) and the percentage of eosinophils in BALF (**Figure 3C**). To assess the role of Panx1 channels in the extracellular

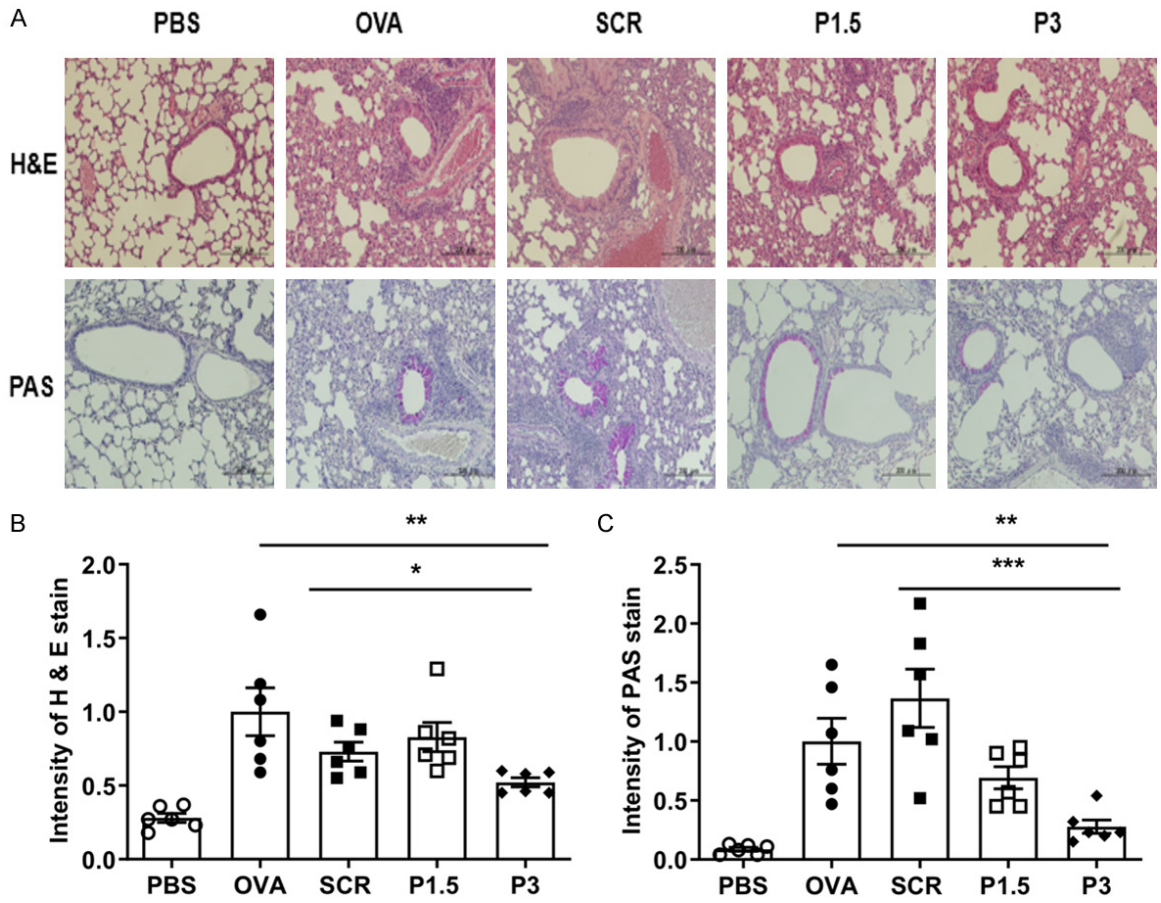

Figure 2. Blockade of Panx1 suppresses airway inflammation and goblet cell hyperplasia in the lungs of OVA-sensitized mice. A. Representative micrographs of hematoxylin and eosin (H&E) stained lung sections to determine eosinophil infiltration (top panel) and periodic acid-Schiff (PAS) stained lung sections to determine goblet cell hyperplasia (bottom panel) (original magnification; 200 ×). Digital images obtained from histological sections were quantified using MetaMorph software. The integrated intensity of H&E and PAS-positive cells was obtained. B. Quantification of H&E-stained cells per bronchia showing airway inflammation. C. Quantification of PAS-stained cells per bronchia showing mucus production. Intensity fold changes were calculated from the average values of the OVA group. PBS, normal control mice; OVA, OVA-sensitized and challenged mice; SCR, 3 mg/kg (scrambled Panx1 peptide) treatment + OVA-sensitized and challenged mice; P1.5 and P3 (1.5 or 3 mg/kg ^{10}Panx1, respectively) treatment + OVA-sensitized and challenged mice. All data are expressed as the mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001.

release of ATP during allergen challenge, we measured ATP levels in mouse BALF. An increase in ATP levels was observed in OVA-sensitized mice compared to PBS control mice. Administration of ^{10}Panx1 reduced the ATP levels in BALF (**Figure 3D**).

Inhibition of Panx1 suppresses the mRNA expression and serum levels of chemokines

Given that we observed reduced numbers of inflammatory cells in the airways of mice treated with ^{10}Panx1, we hypothesized that Panx1 inhibition might impair the expression of key chemokines that promote airway inflammation. Therefore, we analyzed the mRNA expression of CCL11 (eotaxin 1) in lung tissues and serum. Blocking Panx1 with 3 mg/kg of ^{10}Panx1 significantly reduced CCL11 expression in lung tissues (**Figure 4A**) and serum (**Figure 4B**). We also examined whether inhibition of Panx1-mediated ATP release affects CCL2 expression in allergic airway inflammation. Increased mRNA expression of CCL2 was detected in lung tissues and serum from OVA-sensitized mice (**Figure 4C** and **4D**). Blockade of Panx1 significantly reduced the mRNA expression of CCL2 in lung tissues, compared to the SCR con-

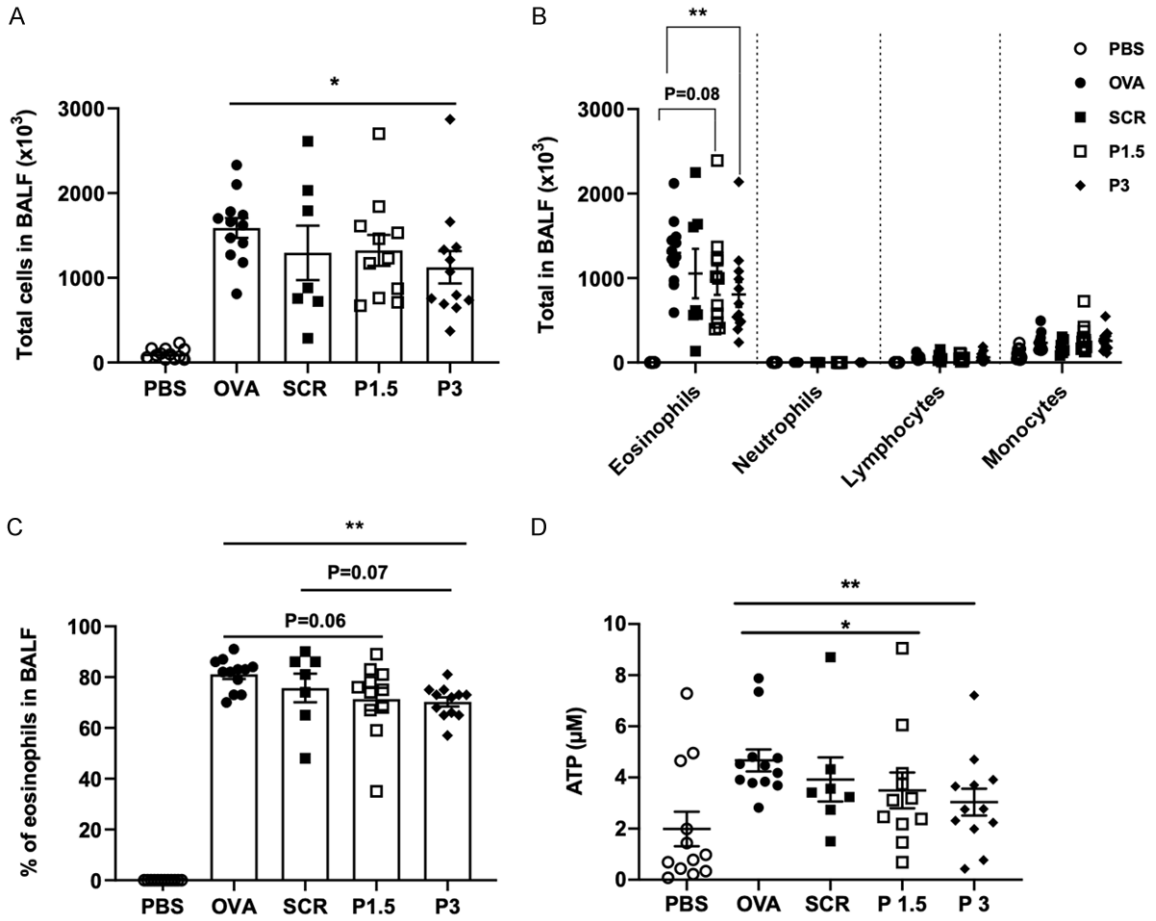

Figure 3. Intratracheal administration of ^{10}Panx1 reduced cell numbers and ATP levels in bronchoalveolar lavage fluid (BALF) of mice. BALF from each mouse was collected to assess cell infiltration in the airways and the accumulation of ATP. A. Total cell counts in BALF were determined using a hemocytometer. B. Differential cell counts were performed according to the morphological characteristics of the cells. C. The percentage of eosinophils in each mouse was determined. D. The level of ATP in each BALF sample was measured by a luminometer. PBS, normal control mice; OVA, OVA-sensitized and challenged mice; SCR, 3 mg/kg (scrambled Panx1 peptide) treatment + OVA-sensitized and challenged mice; P1.5 and P3 (1.5 or 3 mg/kg ^{10}Panx1, respectively) treatment + OVA-sensitized and challenged mice. All data are expressed as the mean ± SEM. *P < 0.05, **P < 0.01.

trol group (**Figure 4C**), but not in serum (**Figure 4D**).

Reduced levels of IL-5 and IL-13 from splenocytes

Because Th2-type cytokines play a critical role in coordinating allergic airway inflammation, we examined the levels of Th2 cytokines in the splenocyte supernatants that were restimulated with OVA for 6 days. Levels of IL-5 and IL-13, but not IL-4, were markedly reduced in P3 group mice (**Figure 5A-C**). Furthermore, we observed a decrease in OVA-specific IgG1 (**Figure 5D**), whereas the level of OVA-specific IgE remained unchanged (**Figure 5E**) in the serum from ^{10}Panx1-treated mice.

Discussion

Extracellular ATP is an important mediator of inflammation in many diseases [17]. For example, airway cells release ATP into the extracellular space upon exposure to allergens [18, 19]. Many cells, including immune cells, airway epithelial cells, alveolar epithelial cells, and airway smooth muscle cells, release ATP into the extracellular milieu [20] under stimuli such as tissue stress or injury [21], leading to recruitment and activation of immune cells [22], such as DCs that are important in allergic responses found in BALF of asthmatic mice [5, 23].

The pannexin1 (Panx1) channel is recognized to be a major conduit for ATP release [24, 25]. A

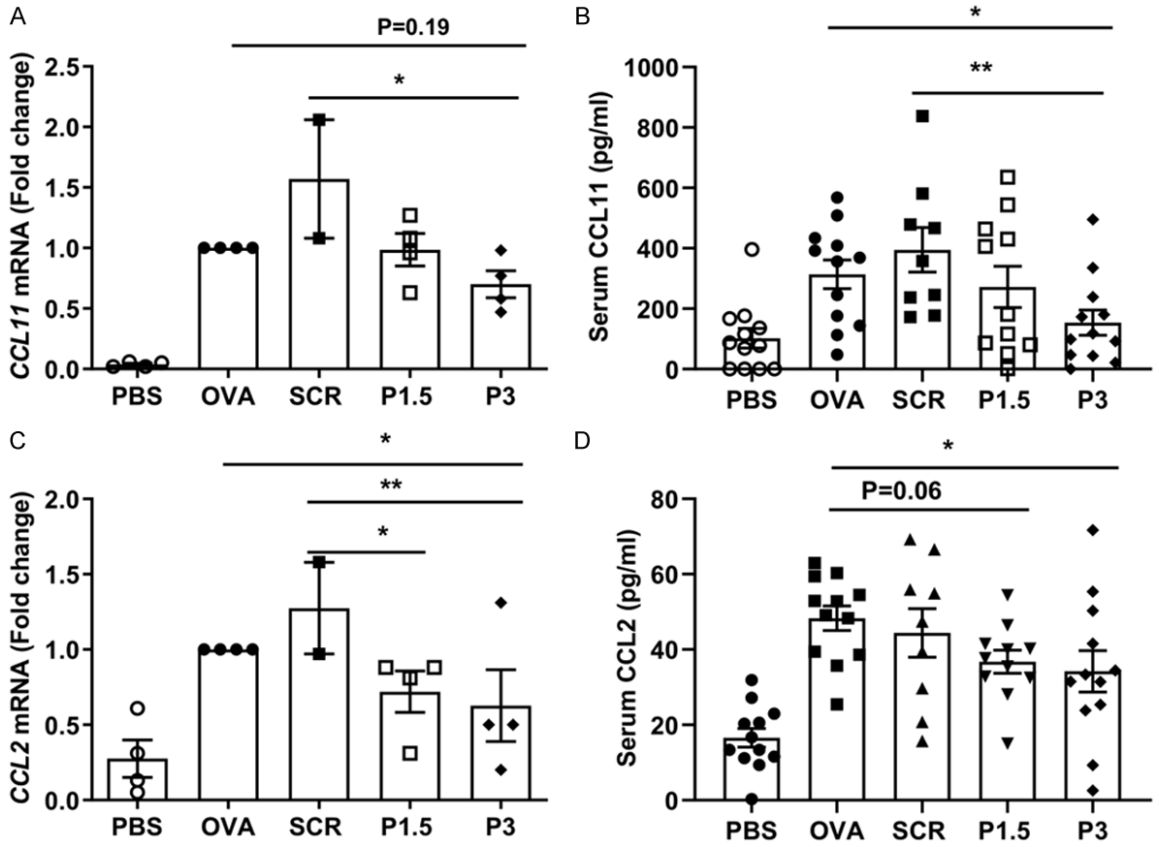

Figure 4. Inhibition of Panx1 suppresses chemokine expression. The mRNA expression of (A) CCL11 and (C) CCL2 was analyzed by real-time PCR. Both genes were normalized to β-actin, and their fold changes were normalized to the OVA-sensitized group. (B) Serum levels of CCL11 and (D) CCL2 were also determined by ELISA (9-12 mice per group). PBS, normal control mice; OVA, OVA-sensitized and challenged mice; SCR, 3 mg/kg (scrambled panx1 peptide) treatment + OVA-sensitized and challenged mice; P1.5 and P3 (1.5 or 3 mg/kg ^{10}Panx1, respectively) treatment + OVA-sensitized and challenged mice. All data are expressed as the mean ± SEM. *P < 0.05, **P < 0.01.

diminished inflammatory response can be induced by targeting extracellular ATP either by hydrolyzing it with apyrase or blocking its release from Panx1 channels with ^{10}Panx1 [5, 23, 26, 27]. Inhibition of ATP signaling by apyrase, blockade, or deletion of P2 receptors leads to lower eosinophil infiltration in lung tissues of murine asthma models [5, 18, 28]. The inhibition of Panx1-mediated ATP release has been shown to attenuate the severity of various pathological and physiological conditions [26, 29-31].

To investigate the potential of Panx1 for targeted treatment of asthma, we used a murine model of OVA-induced asthma. The results showed that inhibition of Panx1 prevented the accumulation of ATP in BALF of asthmatic mice. It significantly reduced eosinophilia and goblet cell hyperplasia and CCL11 and CCL2 mRNA in the lungs of ^{10}Panx1-treated mice. Furthermore, lower levels of IL-5 and IL-13 were detected from splenocytes of ^{10}Panx-treated mice when restimulated with OVA. Overall, these data support ATP as a key mediator of allergic airway inflammation, and targeting Panx1-mediated ATP release can suppress airway inflammation.

Activation of Panx1 can be mediated by the inflammatory cytokine TNF-α [32]. TNF-α plays a crucial role in regulating Panx1 channel opening and leukocyte emigration through the venous endothelium [32], so we also confirmed that the gene expression levels of TNF-α were comparable in the lungs of mice treated without or with ^{10}Panx1 (data not shown). We hypothesized that extracellular ATP exerted its effect via signaling of P2 receptors expressed on immune cells, inducing their activation and chemotaxis toward inflammatory sites [7, 33].

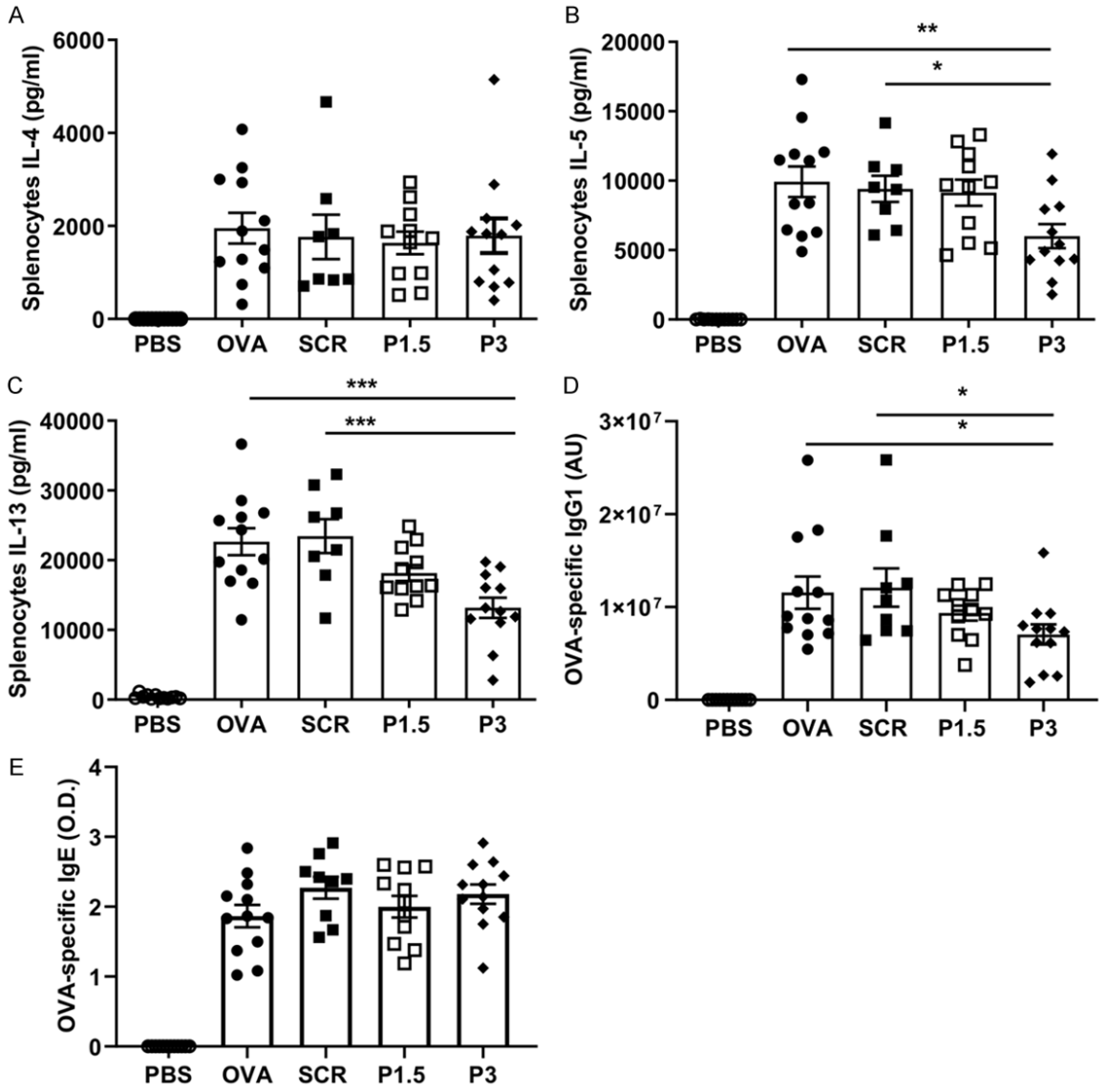

Figure 5. [10]Panx1 administration influences Th2 responses in mice. At sacrifice, splenic cells were isolated and restimulated with 100 μg/ml OVA for six days without [10]Panx1 or scrambled peptide to detect the level of Th2 cytokines. (A) There was no change in IL-4 levels. (B) IL-5 and (C) IL-13 levels were lower in mice after [10]Panx1 administration. The level of OVA-specific antibodies was also determined by ELISA. (D) OVA-specific IgG1 levels were lower in mice treated with [10]Panx1. (E) No change in OVA-specific IgE. PBS, normal control mice; OVA, OVA-sensitized and challenged mice; SCR, 3 mg/kg (scrambled Panx1 peptide) treatment + OVA-sensitized and challenged mice; P1.5 and P3 (1.5 or 3 mg/kg [10]Panx1, respectively) treatment + OVA-sensitized and challenged mice. All data are expressed as the mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001.

Given that we observed a decrease in lung eosinophilia, the accumulation of ATP during allergen exposure may induce the release of chemokines that promote the recruitment of inflammatory cells into the airways [3]. Our data suggest that inhibition of Panx1 reduces mRNA expression and levels of CCL11 and CCL2 in lung tissues. Moreover, *in vitro* stimulation of MLE-12 by exogenous ATP induced CCL2 expression and cellular secretion (Supplementary Figure 1). These findings suggest that ATP may promote airway inflammation through chemokine-mediated recruitment of inflammatory cells into the airways. Studies have demonstrated that CCL2 plays an essential role in asthma by recruiting cells into the airways, and that blocking CCL2 release or signaling reduces airway inflammation, airway re-

modeling, and airway hyperreactivity [34-38]. Thus, a potential mechanism associated with ^{10}Panx1 treatment is that it abolishes Panx1-mediated ATP release and downregulates CCL2 production. However, whether reduced airway inflammation and goblet cell hyperplasia are directly associated with reduced CCL2 levels require further study.

Type 2 immune responses play an important role in the pathophysiology of asthma. They are orchestrated by Th2 cells and innate immune cells by releasing the canonical type 2 cytokines IL-4, IL-5, and IL-13. Among them, IL-4 induces Th2 cell differentiation and promotes antibody gene class switching in B cells, IL-5 promotes eosinophil infiltration, and IL-13 promotes airway remodeling and inflammation [1-3]. Previous studies have found that activation of $CD4^+$ T cells can be prevented by blocking the channel or inhibiting Panx1-mediated P_2X receptor activation [39-41], suggesting that targeting Panx1 may alleviate Th2-mediated allergic responses. We observed decreased levels of IL-5 and IL-13 in the supernatants of splenocytes from ^{10}Panx1-treated mice restimulated with OVA. These observations suggest that treatment with ^{10}Panx1 suppresses cytokines secretion and type 2 immune response, resulting in reduced cellular infiltration of BALF and goblet cell hyperplasia.

Taken together, the results of this study highlight the critical role of Panx1-mediated ATP release in promoting allergic asthma and suggest that inhibition of these channels may serve as a target for reducing inflammatory responses in asthma.

Acknowledgements

This work was supported, in part, by grants from the Ministry of Science and Technology (MOST105-2320-B-182-019-MY3 and MOST 107-2320-B-182-005-MY3) of the Republic of China, as well as from Chang Gung Memorial Hospital (CMRPD1I0081~2, CORPD1F00-21~3, CMRPD160333, and BMRP362). We are grateful to Dr. Albert Ko for English editorial assistance.

Disclosure of conflict of interest

None.

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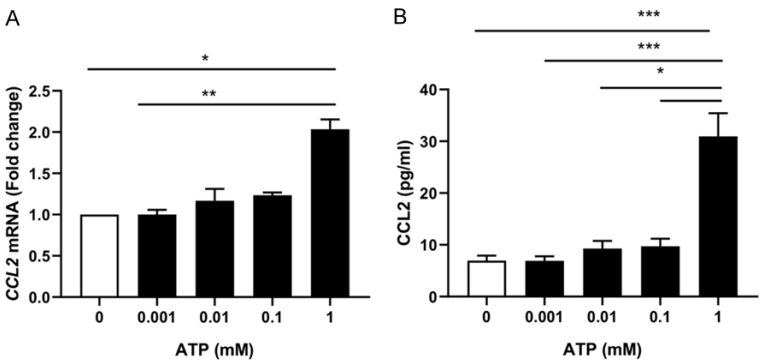

Supplementary Figure 1. Extracellular ATP is a trigger to the production of CCL2. Mouse lung epithelial cell line (MLE-12) was used to determine the cellular response to various concentration of extracellular ATP (0, 0.001, 0.01, 0.1, 1 mM) for 24 hours. CCL2 was upregulated in (A) mRNA and (B) protein levels in a dose-dependent manner. The concentration of CCL2 was assessed by ELISA and the fold change of *ccl2* was performed by real-time PCR. All data are expressed as the mean ± SEM. *P < 0.05.