



ATP/P2X7-NLRP3 axis of dendritic cells participates in the regulation of airway inflammation and hyper-responsiveness in asthma by mediating HMGB1 expression and secretion

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ABSTRACT

The ATP/P2X7 axis of dendritic cells (DCs) mediates the activation of NLRP3 inflammasome and promotes secretion of interleukin (IL) – 1 β and IL-18 to induce T helper (Th) 2, Th17 differentiation in the pathogenesis of asthma. NLRP3 inflammasome also regulates high mobility protein 1 (HMGB1) release in DCs. Recent studies demonstrated the correlation between HMGB1 expression and airway inflammation and hyper-responsiveness (AHR) in asthma. However, the relationship between the ATP/P2X7-NLRP3 axis and HMGB1 in DCs in asthma is still unclear. ATP, apyrase, Brilliant Blue G, BzATP, glibenclamide, and Z-VVAD-FMK were administered to ovalbumin (OVA)-induced murine asthmatic model. For in vitro studies, bone marrow-derived mononuclear cells (BMDCs) were primed with LPS and stimulated with the same reagents. Activation of the ATP/P2X7 axis aggravated airway inflammation and AHR in the lung and induced Th2, Th17 polarization in asthmatic mice. Inhibition of NLRP3 inflammasome weakened cardinal features of asthma and blocked Th2, Th17 polarization. In vitro and in vivo, ATP/P2X7 axis activated NLRP3 inflammasome and induced HMGB1 expression and release from DCs. Inhibition of NLRP3 inflammasome reduced HMGB1 expression and release. The ATP/P2X7-NLRP3 axis of DCs participates in mediating airway inflammation, AHR, and promoting Th2, Th17 inflammatory responses in asthmatic mice by inducing HMGB1 expression and secretion.

1. Introduction

Asthma is a complex chronic airway inflammatory disease caused by a variety of cells and cellular components. Asthma is characterized by the activation of T helper 2 (Th2) cells, which produce Th2 cytokines such as interleukin (IL) – 4, IL-5, and IL-13, infiltration of eosinophils, airway hyper-responsiveness (AHR), and increased levels of serum immunoglobulin (Ig)E [1]. The imbalance of Th1/Th2 is central to the pathogenesis of asthma [1,2]. Recent studies have shown an association between asthma and Th17/Treg imbalance. The imbalance of Th1/Th2 cells may be involved in the pathogenic mechanism of asthma, while the imbalance of Th17/Treg is associated with the worsening of allergic

asthma [2–5].

High mobility protein 1 (HMGB1) is present as a nucleoprotein in all eukaryotic cells and binds to the nuclear chromosome under physiological state, participates in gene transcriptional regulation and cell replication, differentiation, and maturation [6]. It is mainly secreted by macrophages, dendritic cells (DCs), natural killer (NK) cells in response to injury, infection, or other inflammatory stimuli. HMGB1 may also be passively secreted by necrotic cells as alarmins, composed of damage-associated molecular patterns (DAMPs) family with pathogen-associated molecular patterns (PAMPs) [6]. HMGB1 plays an important role as a late inflammatory mediator in the development of sepsis, autoimmune diseases, and other pathological processes [6–8]. HMGB1 is an

Abbreviations: Th, type helper; IL, interleukin; AHR, hyper-responsiveness; HMGB1, high mobility protein 1; DCs, dendritic cells; BMDCs, bone marrow-derived mononuclear cells; DAMPs, damage-associated molecular patterns; PAMP, pathogen-associated molecular patterns; RAGE, advanced glycation end products receptor; TLRs, toll-like receptors; P2X7r, purinergic receptor P2X, ligand-gated ion channel 7 receptor; BALF, bronchial alveolar lavage fluid; MNCs, mononuclear cells; NLRP3, PYD domains-containing protein 3; ASC, apoptosis-associated speck-like protein containing a carboxy-terminal CARD; APCs, antigen-presenting cells; NF- κ B, nuclear factor kappa B

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important protein that regulates many receptors such as advanced glycation end products receptor (RAGE) and toll-like receptors (TLRs) [6,9,10]. It may stimulate the proliferation and migration of neutrophils and monocytes and contribute to DC maturation and adhesion. Furthermore, it may stimulate DCs to secrete more cytokines and pro-inflammatory mediators to help characterize an innate inflammatory response [10–12]. Studies have shown that HMGB1 and endogenous secretory RAGE (esRAGE) levels were significantly augmented in the sputum of asthma patients as compared with the normal controls, and the level of HMGB1 was related to the severity of asthma [13,14]. Numerous studies with animals have shown significantly augmented level of HMGB1 in asthmatic mice as compared with the controls. HMGB1 level was correlated with airway inflammation, increased mucus production, and AHR. HMGB1 promoted the production of factors related with Th2 and Th17 polarization [14–20]. However, its regulatory mechanism in the complex inflammatory network of allergic asthma is questionable.

A large amount of ATP is secreted into the extracellular region in response to tissue damage, stress, immune response, and other pathological conditions. P2X7 receptor (P2X7R) expressed on macrophages, DCs, lymphocytes, and other immune cells mediates the activation of NOD-like receptor family, pyrin domains-containing protein 3 (NLRP3) inflammasome in response to extracellular ATP (eATP) [21]. The process occurs in almost all living cells and regulates a wide range of biological responses [21,22]. NLRP3 inflammasome is mainly composed of NLRP3, adaptor protein apoptosis-associated speck-like protein containing a carboxy-terminal CARD (ASC), and effector protein caspase-1 and expressed in macrophages, DCs, and other immune cells [23]. Several studies have demonstrated that NLRP3 inflammasome activation is critical for the induction and development of allergic airway inflammation in asthma [24–26]. Bruchard et al. [27] confirmed that NLRP3 inflammasome is an important transcriptional regulator in the differentiation of Th2 cells. The activated NLRP3 inflammasome induces pro-caspase-1 cleavage into active caspase-1, which further shear the precursor of IL-1 family members. The active pro-inflammatory factors IL-1 β and IL-18 are released to the extracellular region [23]. IL-1 β promotes the differentiation of Th17 cells and maintains the production of IL-17. In addition, it is crucial for the activation of Th2 cells and synthesis of Th2-related cytokines [24]. IL-18 promotes the secretion of IL-4 and IL-13 by T cells, mast cells, and basophils and enhances the Th2-type immune response [28]. Thus, ATP/P2X7 and NLRP3 inflammasome may form the ATP/P2X7-NLRP3 axis, which is involved in increasing airway inflammation and promoting the differentiation of Th2, Th17 cells in allergic asthma.

The activation of NLRP3 inflammasome was shown to be essential not only for IL-1 β and IL-18 secretion but also for HMGB1 release from the infected and activated immune cells [29–32]. Some studies on infectious diseases have shown infection-induced NLRP3 inflammasome activation, which in turn activates caspase-1 and stimulates IL-1 β secretion. The activated caspase-1 promotes HMGB1 release from the nucleus into the cytoplasm and extracellular region [32,33]. The ATP/P2X7-NLRP3 axis and HMGB1 are all present in DCs, which play an important role as the most potent antigen-presenting cells (APCs) in regulating innate and adaptive immunity by recognizing endogenous and ectogenic antigens and promoting the differentiation of naive CD4⁺ T cells into Th2, Th17 cells, which participate in the pathogenesis of asthma [34,35]. Therefore, we hypothesized that the activation of the ATP/P2X7-NLRP3 axis may promote the release of HMGB1 from the nucleus of DCs into the cytoplasm and extracellular region, wherein it participates in the regulation of airway inflammation, AHR, and differentiation of Th2, Th17 cells in allergic asthma. This may be a new asthmatic intervention treatment target.

2. Materials and methods

2.1. Mice

Female and male C57BL/6 mice (6–8 weeks) were obtained from the Center for Animal Experiment of Wuhan University and maintained in the Animal Biosafety Level 3 Laboratory of Wuhan University (Wuhan, China). All experimental animals were maintained under specific pathogen-free conditions at 25 °C in a 12-h light/dark cycle. All animal protocols were approved by the Animal Welfare Committee of Wuhan University.

2.2. Murine model of allergic asthma

The mice were assigned to 4–5 groups ($n = 4–6$ per group). On days 0 and 14, mice were sensitized with 20 μ g ovalbumin (OVA, Sigma Aldrich, St. Louis, MO, USA) emulsified in 2 mg aluminum hydroxide (Thermo Fisher Scientific Inc, Rockford, IL, USA) in 200 μ L phosphate-buffered saline (PBS) by intraperitoneal (i.p.) injection. On days 21, 22, and 23, mice were challenged with 100 μ g OVA in 50 μ L PBS by intranasal (i.n.) administration. Control mice were sensitized and challenged in the same manner with isometric PBS alone. The mice were executed 24 h after the final challenge and serum, bronchoalveolar lavage fluid (BALF) and lungs were collected for the next step. Apyrase (0.2 IU/g in 40 μ L PBS, i.n., Sigma Aldrich, St. Louis, MO, USA) [36,37], ATP (5 μ g/g in 40 μ L PBS, i.n., Sigma Aldrich, St. Louis, MO, USA), glibenclamide (500 mg/kg in 200 μ L PBS containing 5% dimethyl sulfoxide [DMSO], i.p., Sigma Aldrich, St. Louis, MO, USA) [38,39], caspase-1 inhibitor Z-YVAD-FMK (10 mg/kg in 200 μ L PBS containing 1% DMSO, i.p., Abcam, Cambridge, UK), highly selective agonist for P2X7 receptor BzATP (2'-(3')-O-(4-benzoylbenzoyl)adenosine 5'-triphosphate triethylammonium salt, 50 mg/kg in 200 μ L PBS, i.p., Sigma Aldrich, St. Louis, MO, USA), and highly selective antagonist for P2X7 receptor Brilliant Blue G (BBG, 50 mg/kg in 200 μ L PBS, i.p., Sigma Aldrich, St. Louis, MO, USA) [40–42] were administered to OVA-sensitized mice 30 min before each OVA challenge. Isometric PBS was administered (i.p. or i.n.) to OVA-sensitized mice prior to each OVA challenge as a substitute. Mice were treated with apyrase and BBG 30 min before ATP and BzATP administration, respectively. All experiments were repeated more than three times.

2.3. Lung histopathology

The left lungs were fixed in 4% buffered paraformaldehyde for more than 24 h. The specimens were dehydrated and embedded in paraffin. The lung tissues were cut into 5 μ m sections and stained with hematoxylin-eosin (H&E) and periodic acid-Schiff (PAS) (Baso, Taiwan, China) to evaluate airway inflammation, goblet cell hyperplasia, and mucus secretion. More than four sections were assessed per lung at 200 $\times$ magnification. The peribronchial and perivascular inflammation levels were evaluated by lung inflammatory scores as follows: 0, normal; 1, few inflammatory cells; 2, a ring of inflammatory cells (1–5-layer deep); and 3, a ring of inflammatory cells (> 5-layer deep). Goblet cells were counted by the number of PAS-positive cells in the airway epithelium using Image Pro Plus 6.0 (IPP 6.0) software to determine airway basement membrane perimeter (Pbm) and PAS-positive area of airway epithelium (Apas⁺) for the calculation of Apas⁺/Pbm.

2.4. Immunohistochemistry

For immunohistochemistry of HMGB1, left lung sections were de-paraffinized, rehydrated, and submerged. DCs were seeded onto chamber slides and fixed. All lung tissue sections and fixed DCs were incubated in 1:100 dilution of polyclonal rabbit anti-HMGB1 antibody (Cell Signaling Technology, Beverly, MA, USA), followed by their treatment with horseradish peroxidase (HRP)-linked anti-rabbit

secondary antibody (goat anti-Rabbit IgG [H+L]-HRP, Bioworld Technology, St. Louis, MN, USA). These were stained with diaminobenzidine (DAB) (Vectastain ABC elite kit; Vector Laboratories, Burlingame, CA, USA). HMGB1-positive expression was localized in the nucleus, cytoplasm, or extracellular region as claybank. IPP 6.0 software was applied for image analysis.

2.5. Collection of bronchoalveolar lavage fluid (BALF)

We collected BALF to analyze cytokines in supernatants and cellular components. At 24 h after the final OVA challenge, mice were sacrificed after collecting blood by eyeball picking. The supernatant of BALF was collected and stored at -80°C for the analysis of cytokine levels. Slides for the cellular component were prepared by cytocentrifugation (TXD3 cytocentrifuge, Xiangyi, Changsha, China) and stained with Wright-Giemsa Stain kit (Jiancheng Bioengineering Institute, Nanjing, China). At least 400 cells were counted for each slide. The number of eosinophils, macrophages, neutrophils, and lymphocytes was expressed as the absolute number from the total cells count.

2.6. Airway hyper-responsiveness (AHR)

At 24 h after the final OVA challenge, AHR to methacholine was assessed using a small animal lung function instrument (Buxco Electronics, Troy, NY, USA). Briefly, mice were i.p. anesthetized with 1% Nembutal, intubated, and challenged with vehicle (PBS), followed by the treatment with an increasing concentration of methacholine (3.125, 6.25, 12.5, 25, and 50 mg/mL). Resistance index (RI) and dynamic compliance (C_{dyn}) were automatically calculated by the instrument.

2.7. Generation of bone marrow-derived dendritic cells (BMDCs)

Male C57BL/6 mice (5–8 weeks) were sacrificed and BMDCs prepared from femur bone marrow suspensions. The cells were cultured at a density of $1-2 \times 10^6$ cells/mL in Roswell Park Memorial Institute (RPMI)–1640 medium supplemented with 10% fetal bovine serum, 0.1% $100\times$ penicillin-streptomycin solution, 10 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF; PeproTech, Rocky Hill, NJ, USA), and 10 ng/mL recombinant mouse IL-4 (PeproTech, Rocky Hill, NJ, USA) at 37°C in a 5% CO_2 incubator for 7 days. The medium was replaced on days 3 and 5. The purity of the immature DCs was at least 90%. On days 7, BMDCs were stimulated with lipopolysaccharide (LPS, 1 $\mu\text{g/mL}$, Sigma Aldrich, St. Louis, MO, USA), glibenclamide (50 $\mu\text{g/mL}$), and Z-YVAD-FMK (100 μM) for 24 h [43,44]. After LPS stimulation, cells were treated with apyrase (10 IU/mL) for 30 min, followed by their treatment with ATP (3 mM) for an additional 2 h [45,46]. BMDCs and the medium supernatant were collected for subsequent real-time polymerase chain reaction (RT-PCR), western blot, and enzyme-linked immunosorbent assay (ELISA) analyses.

2.8. RNA extraction and real-time polymerase chain reaction (RT-PCR)

Total RNA was extracted from the right lung tissue and BMDCs by Trizol reagent (Invitrogen/Thermo Fisher Scientific Inc., Carlsbad, CA, USA). Complementary DNA (cDNA) synthesis was performed using ReverTra Ace qPCR RT kit (Toyobo CO., LTD., Tokyo, Japan) according to the manufacturer's protocol. The target genes were measured using RT-PCR with SYBR Premix Ex Taq™ (Takara Bio Inc., Otsu, Japan). The primers were obtained from Sangon Biotech (Shanghai, China) and tested for sequence specificity using the Primer-BLAST at the National Center for Biotechnology Information (NCBI). All primer sequences of target gene are listed in Table 1. The amplification of genomic DNA was performed in QuantStudio 6 Flex (ABI Life Technology, USA). The relative expression of the target gene was calculated using the comparative $2^{-\Delta\Delta\text{Ct}}$ method with glyceraldehyde 3-phosphate

Table 1
Primer sequences and product sizes.

Gene name	Primer sequences (5'-3')	Product size (bp)
GATA3		239
Forward:	5'- CTGGAGGAGGAACGCTAATG -3'	239
Reversed:	5'- AGATGTGGCTCAGGGATGAC -3'	
T-bet		137
Forward:	5'- CCATTCTGTCTTCACCG -3'	
Reversed:	5'- CTGCCTTCTGCCTTTCAC -3'	
ROR γ t		221
Forward:	5'-GCGGCTTTCAGGCTTCATGGAG-3'	
Reversed:	5'-GGGCGCTGAGGAAGTGGGAAA-3'	
FoxP3		181
Forward:	5'-ACTCGCATGTTTCGCCTACTT-3'	
Reversed:	5'-AGGGATTGGAGCACTTGTG-3'	
NLRP3		237
Forward:	5'-GACCAGCCAGAGGTGGAATGA-3'	
Reversed:	5'-CTGCGTGTAGCGCACTGTTGA-3'	
ASC		121
Forward:	5'-CACCAGCCAAGACAAGATGA-3'	
Reversed:	5'-CTCCAGGTCCATCACCAGT-3'	
Caspase-1		190
Forward:	5'-AACAGAACAAAGAAGATGGCACA-3'	
Reversed:	5'-CCAACCTCGGAGAAAGAT-3'	
HMGB1		112
Forward:	5'-GCGAGCATCTGGCTTATC-3'	
Reversed:	5'-TTCAGCTTGGCAGCTTCT-3'	
P2X7		145
Forward:	5'-ATACCACGAGAAACATCTTGCC-3'	
Reversed:	5'-ATTCTCCCTGAACATGCCACC-3'	
GAPDH		150
Forward:	5'-TGTGTCCGTCGTGGATCTGA-3'	
Reversed:	5'-TTGCTGTTGAAGTCGAGGAG-3'	

GATA3, GATA binding protein 3; T-bet, T-box transcription factor; RoR γ t, retinoic acid receptor-related orphan receptor γ t; FoxP3, forkhead box protein 3; NLRP3, NOD-like receptor family, pyrin domain-containing 3; ASC, apoptosis-associated speck-like protein containing a carboxy-terminal CARD; HMGB1, high mobility group box 1; P2X7, purinergic receptor P2X, ligand-gated ion channel 7; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

dehydrogenase (GAPDH) as the internal reference gene.

2.9. Western blot analysis

The total protein was extracted from the right lung. After detection of protein concentration, 40 μg of total protein was separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and then transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore Corp., Billerica, MA, USA). The membranes were blocked with 5% non-fat milk in TBST (Tris-buffered saline containing Tween 20) for 2 h at room temperature on a shaking instrument. The PVDF membranes were overnight incubated with diluted primary antibodies (1:1000) against NLRP3, ASC (Cell Signaling Technology, Danvers, MA, USA), caspase-1, P2X7R (Novus Biologicals, Littleton, CO, USA), HMGB1 antibody (Cell Signaling Technology, Beverly, MA, USA), pro-caspase-1 (Abcam, Cambridge, UK) at 4°C and treated with diluted HRP-conjugated secondary anti-rabbit antibody (1:50000, BOSTER Biological Technology Co., Wuhan, China) at 37°C for 2 h. Chemiluminescence images were captured with Pierce enhanced chemiluminescence western blotting substrate (Thermo Fisher Scientific, Waltham, MA, USA) using BandScan 5.0 (Glyko, Novato, CA, USA) gel image software.

2.10. Enzyme-linked immunosorbent assay (ELISA)

The levels of IL-4, IL-5, IL-13, interferon gamma (IFN- γ), IL-10, IL-17A, and IL-22 in BALF were detected using ELISA kits (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions. The total IgE and OVA-specific IgE in serum were measured using specific ELISA kits (eBioscience, San Diego, CA, USA). HMGB1 levels in BALF,

serum, and cell culture supernatant were detected using an ELISA kit (LifeSpan BioSciences, Inc. WA, USA). The concentration of cytokines was expressed as picogram per milliliter (pg/mL), while that of HMGB1, total IgE, and OVA-specific IgE was expressed as nanogram per milliliter (ng/mL).

2.11. Flow cytometric analysis

For the determination of Th1, Th2, Th17, and Treg cell percentage of CD4⁺ Th cells in lungs, mouse lung mononuclear cells (MNCs) were prepared at a density of 2×10^6 cells/mL and cultured for 2 h with 500 ng/mL ionomycin (Tocris Bioscience, Bristol, UK) and 20 ng/mL phorbol 12-myristate 13-acetate (PMA, Tocris Bioscience, Bristol, UK). The cells were blocked with $1 \times$ brefeldin A (Biolegend, San Diego, CA, USA) for 4 h and stained with fluorescein isothiocyanate (FITC)-CD3e and phycoerythrin (PE)-cy7-CD4 (eBioscience, San Diego, CA, USA). CD4⁺ Th cells, except Treg cells, were measured as CD3e and CD4 double-positive cells. After fixation and permeabilization (intracellular fixation and permeabilization buffer set, eBioscience, San Diego, CA, USA), CD4⁺ Th cells were labeled with the following antibodies for intracellular staining: PE-IFN- γ , PE-IL-4, and PE-IL-17A (eBioscience, San Diego, CA, USA). For Treg cells, MNCs were labeled double positively with PE-cy7-CD4 and PE-CD25 (eBioscience, San Diego, CA, USA) and then fixed and permeabilized (forkhead box P3 [FoxP3] fixation/permeabilization concentrate and diluent, eBioscience, San Diego, CA, USA), followed by their staining for intranuclear region with FITC-FoxP3 (eBioscience, San Diego, CA, USA). All cells were analyzed by flow cytometry (FACSaria III, BD Biosciences, USA).

2.12. Statistical analyses

All data are expressed as mean $\pm$ standard deviation (SD). All experiments were repeated more than thrice ($n = 4$ –6 mice per group). Presented data are one representative experiment of three repeats. The differences were compared using one-way analysis of variance (ANOVA). Data were analyzed using the GraphPad Prism 5 (GraphPad Software Inc, San Diego, CA, USA) software and SPSS 22.0 software (IBM SPSS, Chicago, IL, USA). $P < 0.05$ was considered statistically significant. *, **, and *** denote $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

3. Results

3.1. The activation of ATP/P2X7 axis aggravated airway inflammation and mucus production in the lung tissue and increased AHR to methacholine in asthmatic mice

To further characterize ATP/P2X7 axis in an OVA-induced mouse model of asthma, at days 21–23, we exogenously supplemented OVA-sensitized mice with ATP and apyrase (an extracellular ATP scavenger) 30 min before each OVA challenge to change extracellular ATP levels. P2X7 receptor was intervened with the administration of its selective antagonist (BBG) and agonist (BzATP) 30 min before each OVA challenge. The detailed protocol of OVA sensitization/challenge and timeline of ATP, apyrase, BBG and BzATP treatment are shown in Fig. 1a. OVA-induced asthmatic mice showed typical pathological features such as infiltration of lungs with inflammatory cells and goblet cell hyperplasia (Fig. 1b, d). ATP and BzATP aggravated the peribronchial and perivascular inflammation and mucus production in the lung tissue. On the other hand, apyrase and BBG had the opposite effect. No significant difference was observed in the number of PAS-positive cells between BBG-treated and PBS-treated asthma mice (Fig. 1b, c, d, e). The different inflammatory cells (total cells, eosinophils, and lymphocytes) observed in BALF were in accordance with the histopathological evidences (Fig. 1f, h). Increased levels of total IgE and OVA-specific IgE were observed in the serum of the asthmatic mice treated with ATP and

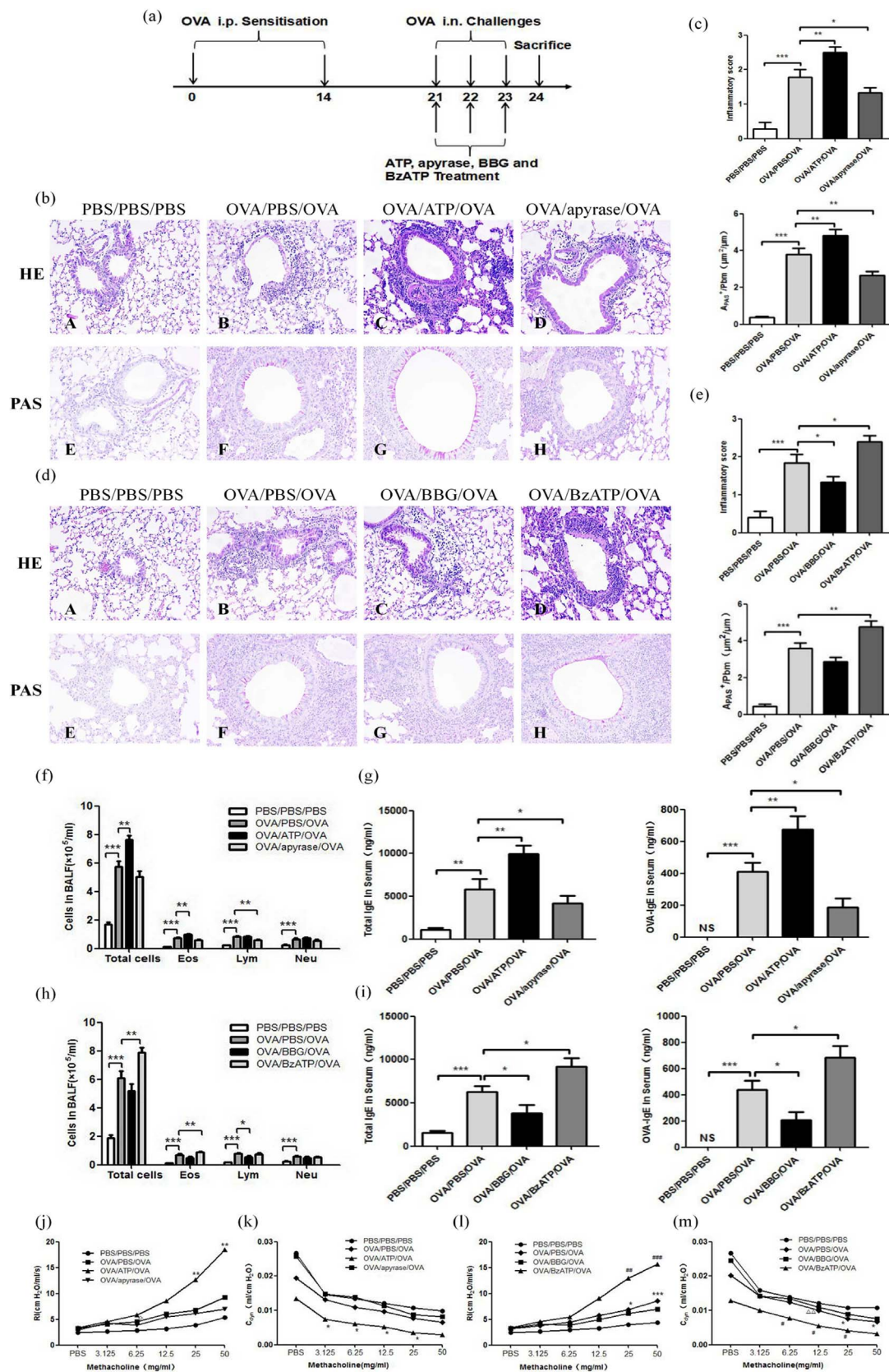
BzATP, while their levels were decreased in asthmatic mice administered with apyrase and BBG (Fig. 1g, i). To evaluate AHR, we analyzed the changes in RI and Cdyn in mice treated with different concentrations of methacholine. The results of RI revealed statistically significant differences at high concentration in asthmatic mice treated with ATP as compared with control mice (Fig. 1j). In another set of experiment, we observed statistical differences at high concentration between BzATP treatment group and PBS-treated group, PBS-treated group and control group (Fig. 1l). In comparison with the PBS-treated asthmatic group, ATP-treated asthmatic group showed a marked reduction in the compliance of lung between 3.125 and 25 mg/mL concentrations (Fig. 1k). Furthermore, We found significant difference between PBS-treated asthma group and control group at 25, 50 mg/mL concentration of methacholine, statistical differences were also observed between BzATP-treated and PBS-treated asthma group at 6.25–25 mg/mL concentration (Fig. 1m). These results suggest that either the administration of exogenous ATP or activation of P2X7 receptor may aggravate airway inflammation and mucus production in the lung tissue and increase AHR to methacholine in asthmatic mice.

3.2. The activation of ATP/P2X7 axis induced Th2, Th17 inflammatory responses in OVA-sensitized and challenged asthma model

To investigate the role of ATP/P2X7 axis in Th2, Th17 inflammatory responses in OVA-induced murine asthma model, The whole left lungs from different groups were digested 24 h after the final OVA challenge. Isolated lung MNCs were subsequently stained for CD3e, CD4 and CD25 and analyzed by flow cytometry. CD4⁺ Th cells except Treg were marked as CD3e and CD4 double-positive cells, Treg were marked as CD4⁺CD25⁺FoxP3⁺ cells. We analyzed percentages of CD4⁺Th cells expressing IFN- γ ⁺ (Th1), IL-4⁺ (Th2), IL-17A⁺ (Th17), and FoxP3⁺ (Treg) in CD4⁺ Th cells, levels of cytokines mediated by Th2 (IL-4, IL-5, and IL-13), Th1 (IFN- γ), Th17 (IL-17A and IL-22), and Treg (IL-10) in BALF and mRNA expressions of key transcription factors related to Th2 (GATA3) and Th17 (ROR γ t) as well as Th1 (T-bet) and Treg (FoxP3) in the lung tissue. In comparison with the control group, all asthmatic groups showed a significant increase in the percentage of CD4⁺ Th cells expressing IL-4⁺ and IL-17A⁺ (Fig. 2a, b, d, e) and a rise in the mRNA expression of corresponding transcription factors GATA3 and ROR γ t (Fig. 2c, f). In addition, these groups showed an increase in levels of IL-4, IL-5, IL-13, IL-17A, and IL-22 in BALF (Fig. 2g, h) as compared with the control group. On the other hand, a decrease in the percentage of CD4⁺ Th cells expressing IFN- γ ⁺ and FoxP3⁺ (Fig. 2a, b, d, e) with an accompanied fall in the expression of T-bet and FoxP3 mRNA (Fig. 2c, f) and IFN- γ and IL-10 levels (Fig. 2g, h) was observed in the asthmatic groups as compared with the control group. These changes were further augmented in ATP- and BzATP-treated asthmatic groups as compared with PBS-treated asthma group. Apyrase and BBG treatment lowered these effects (Fig. 2a, b, c, d, e, f, g, h). Thus, the activation of ATP/P2X7 axis may induce Th2, Th17 differentiation and increase the production of related cytokines and mRNA expression of transcription factors, while the inhibition of ATP/P2X7 axis may block Th2, Th17 differentiation and reduce the production of related cytokines and mRNAs of transcription factors.

3.3. The inhibition of NLRP3 inflammasome weakened airway inflammation and mucus production in the lung tissue and reduced AHR to methacholine in asthmatic mice

To demonstrate the role of NLRP3 inflammasome in OVA-sensitized and challenged asthma model, at days 21–23, we i.p. injected OVA-sensitized mice with NLRP3 inhibitor (glibenclamide) and caspase-1 inhibitor (Z-YVAD-FMK) 30 min before OVA challenge. The detailed protocol of OVA sensitization/challenge and timeline of glibenclamide and Z-YVAD-FMK administration are shown in Fig. 3a. Glibenclamide and Z-YVAD-FMK treatment markedly decreased the infiltration of



(caption on next page)

Fig. 1. Increased allergic airway inflammatory response and AHR by activating ATP/P2X7 axis in OVA-induced asthmatic mice. All groups were marked as sensitization/treatment/challenge. (a) Experimental protocols of administration of ATP, apyrase, BBG, BzATP to OVA-immunized and challenged murine asthmatic model are shown. (b: A-D, d: A-D) Micrographs of HE staining of lung tissue sections (200 $\times$ magnification). (b: E-H, d: E-H) Micrographs of PAS staining of lung tissue sections (200 $\times$ magnification). (c, e) Lung inflammatory score and Apas⁺/Pbm are shown. (f, h) The number of total inflammatory cells, eosinophils, lymphocytes, and neutrophils was measured in BALF. (g, i) Total IgE and OVA-specific IgE levels in serum were analyzed by ELISA. (j, l) Resistance index (RI) and (k, m) dynamic compliance (Cdyn) to double multiplication concentration of methacholine (3.125, 6.25, 12.5, 25, and 50 mg/mL) and PBS were measured. (j, k) $^{\circ}$ OVA/ATP/OVA versus OVA/PBS/OVA, $^{\circ}$ P < 0.05, ** P < 0.01. $^{\diamond}$ OVA/PBS/OVA versus PBS/PBS/PBS, $^{\diamond}$ P < 0.05. (l, m) $^{\circ}$ OVA/PBS/OVA versus PBS/PBS/PBS, $^{\circ}$ P < 0.05, ** P < 0.001. $^{\#}$ OVA/BzATP/OVA versus OVA/PBS/OVA, $^{\#}$ P < 0.05, $^{##}$ P < 0.01, $^{###}$ P < 0.001. All data are represented as mean $\pm$ SEM (n = 4–6 mice per group). ND (not detected), $^{\circ}$ P < 0.05, ** P < 0.01, *** P < 0.001.

inflammatory cells and reduced the number of goblet cells in the lung (Fig. 3b, c). In comparison with PBS-treated asthma group, glibenclamide- and Z-YVAD-FMK-treated asthma group showed a decrease in the number of total inflammatory cells, eosinophils, lymphocytes, and neutrophils in BALF (Fig. 3d). In accordance with these results, the level of total IgE and OVA-specific IgE in the serum also decreased in glibenclamide and Z-YVAD-FMK treatment groups (Fig. 3e). However, the combination treatment failed to exert statistically significant superimposed effects on the reduction in airway inflammation of the lung tissue in asthmatic mice. However, significant differences in airway resistance and compliance were observed between OVA-induced asthma group and control group. At 25 and 50 mg/mL concentration of methacholine, significant differences in RI and Cdyn were observed in glibenclamide and Z-YVAD-FMK treatment groups as compared with PBS-treated asthma group (Fig. 3f, g). Overall, these data indicate that the inhibition of NLRP3 inflammasome may weaken the airway inflammation and mucus production in the lung tissue and reduce AHR to methacholine in asthmatic mice.

3.4. The inhibition of NLRP3 inflammasome blocked Th2, Th17 inflammatory responses in OVA-sensitized and challenged asthma model

We verified whether NLRP3 inflammasome complex regulates Th2, Th17 inflammatory response in the asthma model. The whole left lungs from different groups were digested 24 h after the final OVA challenge. Flow cytometric analysis of lung MNCs revealed that glibenclamide and Z-YVAD-FMK treatment reduced the percentage of CD4⁺ Th cells expressing IL-4⁺ and IL-17A⁺ and augmented the percentage of CD4⁺ Th cells expressing IFN- γ ⁺ and FoxP3⁺ as compared with the PBS treatment (Fig. 4a, b). This change was accompanied with a decrease in GATA3 and ROR γ t mRNA expression and an increase in T-bet and FoxP3 mRNA expression in the lung tissue (Fig. 4c). In comparison with PBS treatment, glibenclamide and Z-YVAD-FMK treatment effectively decreased the levels of IL-4, IL-5, IL-13, IL-17A, and IL-22 and increased the levels of IFN- γ and IL-10 (Fig. 4d). However, glibenclamide and Z-YVAD-FMK combination treatment failed to show a statistically significant composite effect in comparison with the single treatment. These results show that the inhibition of NLRP3 inflammasome may block Th2, Th17 inflammatory responses in OVA-sensitized and challenged asthma model.

3.5. ATP/P2X7 axis activated NLRP3 inflammasome in DCs

We have confirmed that either ATP/P2X7 axis or NLRP3 inflammasome participate in the regulation of airway inflammation and AHR and induce Th2, Th17 inflammatory responses in the asthma model. To further explore the regulatory mechanism of ATP/P2X7 axis and NLRP3 inflammasome in asthma, we evaluated if ATP/P2X7 axis may regulate NLRP3 inflammasome in DCs and promote the polarization of Th2, Th17 cells for their participation in the pathogenesis of asthma. Right lungs from different groups were exteriorised 24 h after the final OVA challenge for Western blot and RT-PCR analysis. Western blot analysis for P2X7R expression revealed that ATP and BzATP treatment increased the expression of P2X7R, while apyrase and BBG treatment reduced P2X7R levels as compared with PBS-treated group in the lung tissue of asthmatic mice (Fig. 5a, b). In vitro, we exogenously supplemented ATP and found a remarkable increase in the expression

of P2X7R protein (Fig. 5c) and mRNA (Fig. 5g) in LPS-primed BMDCs; this effect was attenuated by apyrase (Fig. 5c, g). Extracellular ATP is generally considered as the prototypical danger signal. Thus, ATP interacts with P2X7R expressed by DCs to form ATP/P2X7 axis and participate in the regulation of Th2, Th17 inflammatory responses in asthma. NLRP3 inflammasome comprises a proteolytic complex formed by three key components, including NLRP3 (functions as a pattern recognition receptor), adaptor protein ASC, and caspase-1. The expression of NLRP3, ASC, and caspase-1 was increased following administration of ATP and BzATP as compared with control, while the administration of apyrase and BBG reduced their expression levels (Fig. 5d, e). Similar results were observed with in vitro experiments; western blot (Fig. 5f) and RT-PCR (Fig. 5h) analyses showed that ATP administration effectively increased the expression of NLRP3, ASC, and caspase-1 and their corresponding mRNAs in LPS-primed BMDCs, while apyrase abrogated this effect (Fig. 5f, h). Upon formation of NLRP3 inflammasome complex, caspase-1 is activated after its cleavage from pro-caspase-1. As pro-caspase-1 is the precursor of caspase-1, no statistical difference was detected in pro-caspase-1 level between different groups (Fig. 5d, e, f). These results suggest that eATP is the activator of P2X7R expressed by DCs and that ATP/P2X7 axis in DCs activates NLRP3 inflammasome complex. The resulting ATP/P2X7-NLRP3 axis in DCs participates in the allergic airway inflammation, AHR, and polarization of Th2 and Th17 cells in asthma.

3.6. ATP/P2X7 axis induced HMGB1 expression and release in DCs

To further explore the mechanism underlying the effect of ATP/P2X7 axis in DCs, we evaluated the relationship between ATP/P2X7 axis and HMGB1. HMGB1 located in the cytoplasm and nucleus is also secreted by mature DCs in response to inflammatory stimuli [6–8]. Immunohistochemical analysis of the lung tissue revealed that HMGB1 protein was primarily located in the nucleus and cytoplasm of inflammatory and epithelial cells and its positive expression was clay-bank. The lung tissue from the control group showed low expression level of HMGB1, while the lung tissue from the OVA-induced asthma group showed strong expression level of HMGB1. The number of HMGB1-positive cells was significantly higher in ATP and BzATP treatment groups as compared with the asthma group, while HMGB1-positive cell number was low in apyrase and BBG treatment groups. Statistical difference was reported among all the treatment groups (Fig. 6a, b, c, d), consistent with the results of western blot (Fig. 6e, f). We also tested the levels of HMGB1 in serum and BALF by ELISA (Fig. 6g, h) and found that HMGB1 secreted into the extracellular space increased following ATP and BzATP administration, while apyrase and BBG administration reduced the level of HMGB1 in asthmatic mice. Immunohistochemical analysis of BMDCs showed that ATP stimulated HMGB1 expression in LPS-primed BMDCs, while apyrase reduced HMGB1 expression in LPS-primed BMDCs and ablated ATP-induced HMGB1 expression (Fig. 6i, j), as observed with western blot (Fig. 6k) and RT-PCR (Fig. 6l) analyses. The analysis of BMDC culture supernatant revealed that ATP not only induced the expression of HMGB1 but also stimulated its release from BMDCs; apyrase treatment reduced HMGB1 release into intercellular spaces (Fig. 6m). Overall, the activation ATP/P2X7 axis may increase the expression of HMGB1 in the cytoplasm and nucleus of DCs and induce HMGB1 release from DCs into the extracellular region. The inhibition of ATP/P2X7 axis, on the other

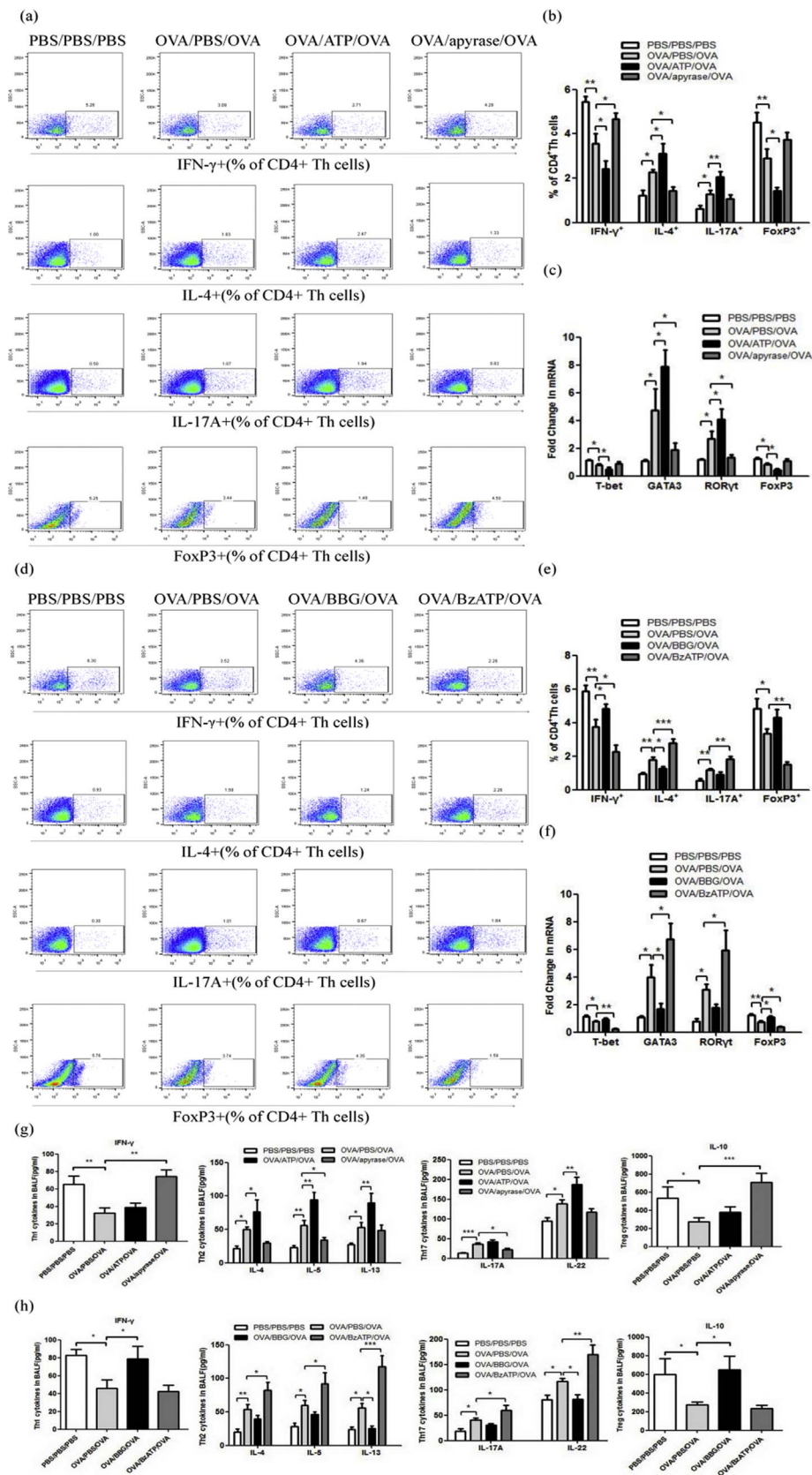


Fig. 2. Enhanced Th2, Th17 differentiation by activating ATP/P2X7 axis in the lung tissue of OVA-induced asthmatic mice. All groups were marked as sensitization/treatment/challenge. (a, d) IFN- γ ⁺, IL-4⁺, IL-17A⁺, FoxP3⁺ Th cells were examined from CD4⁺ (Th) cells by flow cytometric analysis. (b, e) The percentage of CD4⁺ Th cells expressing IFN- γ ⁺, IL-4⁺, IL-17A⁺, and FoxP3⁺ in the left lung CD4⁺ Th cells. (c, f) The transcription factors T-bet (Th1 cells), GATA3 (Th2 cells), ROR γ t (Th17 cells), and FoxP3 (Treg) were measured in the right lung. (g, h) Th2-associated cytokines (IL-4, IL-5, and IL-13), Th1-associated cytokines (IFN- γ), Th17-associated cytokines (IL-17A and IL-22), and Treg-associated cytokines (IL-10) were measured in BALF by ELISA. All data are represented as mean $\pm$ SEM (n = 4–6 mice per group). *P < 0.05, **P < 0.01, and ***P < 0.001.

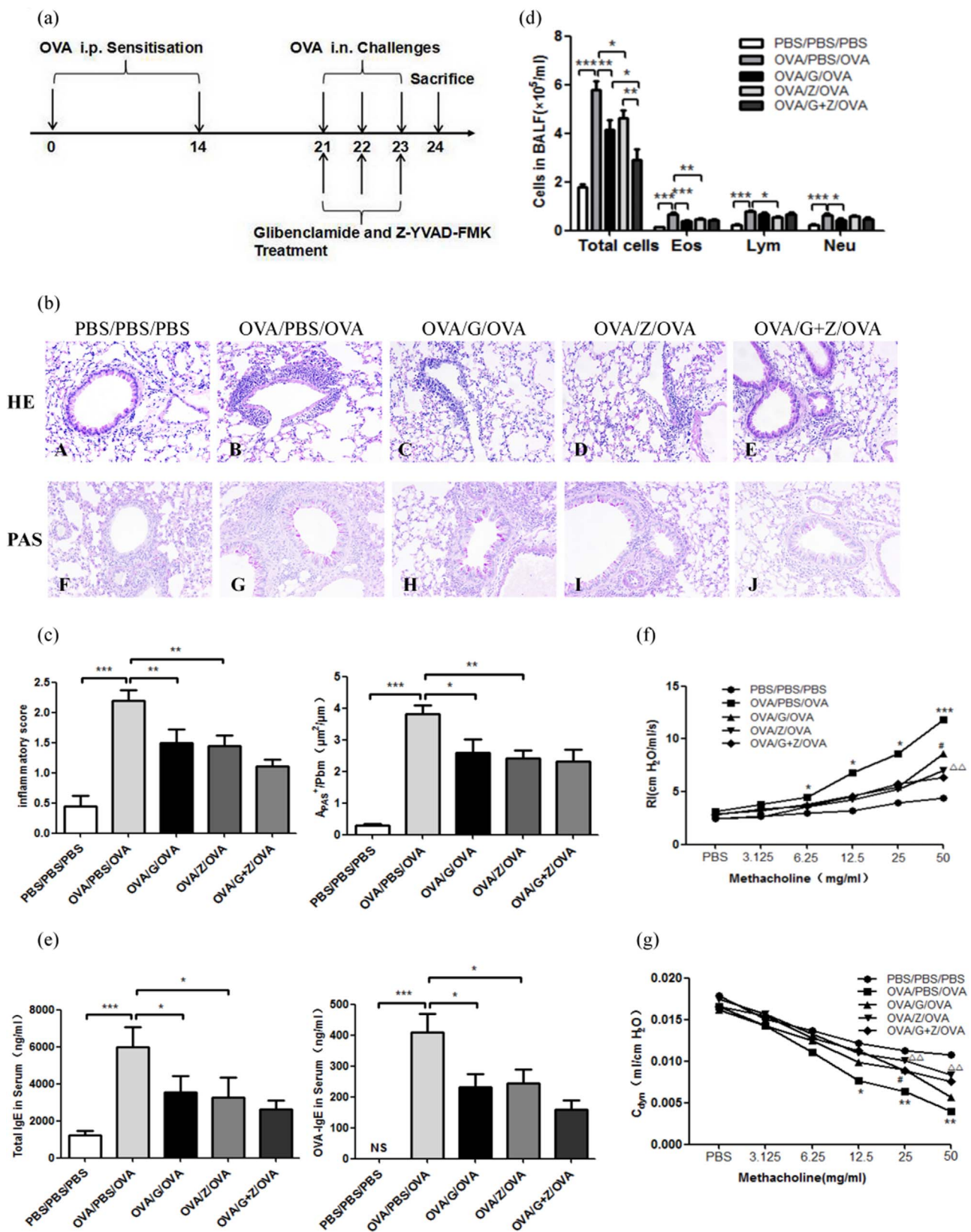


Fig. 3. Reduced allergic airway inflammatory response and AHR by blocking NLRP3 inflammasome in OVA-induced asthmatic mice. All groups were marked as sensitization/treatment/challenge (G = glibenclamide and Z = Z-YVAD-FMK). (a) Experimental protocols of administration of glibenclamide and Z-YVAD-FMK to OVA-immunized and challenged murine asthmatic model are shown. (b) Micrographs of HE and PAS staining of the lung tissue sections (200× magnification). (c) Lung inflammatory score and Apas⁺/Pbm are shown (d) The number of total inflammatory cells, eosinophils, lymphocytes, and neutrophils was measured in BALF. (e) Total IgE and OVA-specific IgE levels in serum were analyzed by ELISA. (f) Resistance index (RI) and (g) dynamic compliance (C_{dyn}) to double multiplication concentration of methacholine (3.125, 6.25, 12.5, 25, and 50 mg/mL) and PBS were measured. *OVA/PBS/OVA versus PBS/PBS/PBS, *P < 0.05, **P < 0.01, ***P < 0.001. #OVA/G/OVA versus OVA/PBS/OVA, #P < 0.05. △OVA/Z/OVA versus OVA/PBS/OVA, △P < 0.05, △△P < 0.01. All data are represented as mean ± SEM (n = 4–6 mice per group). ND (not detected), *P < 0.05, **P < 0.01, ***P < 0.001.

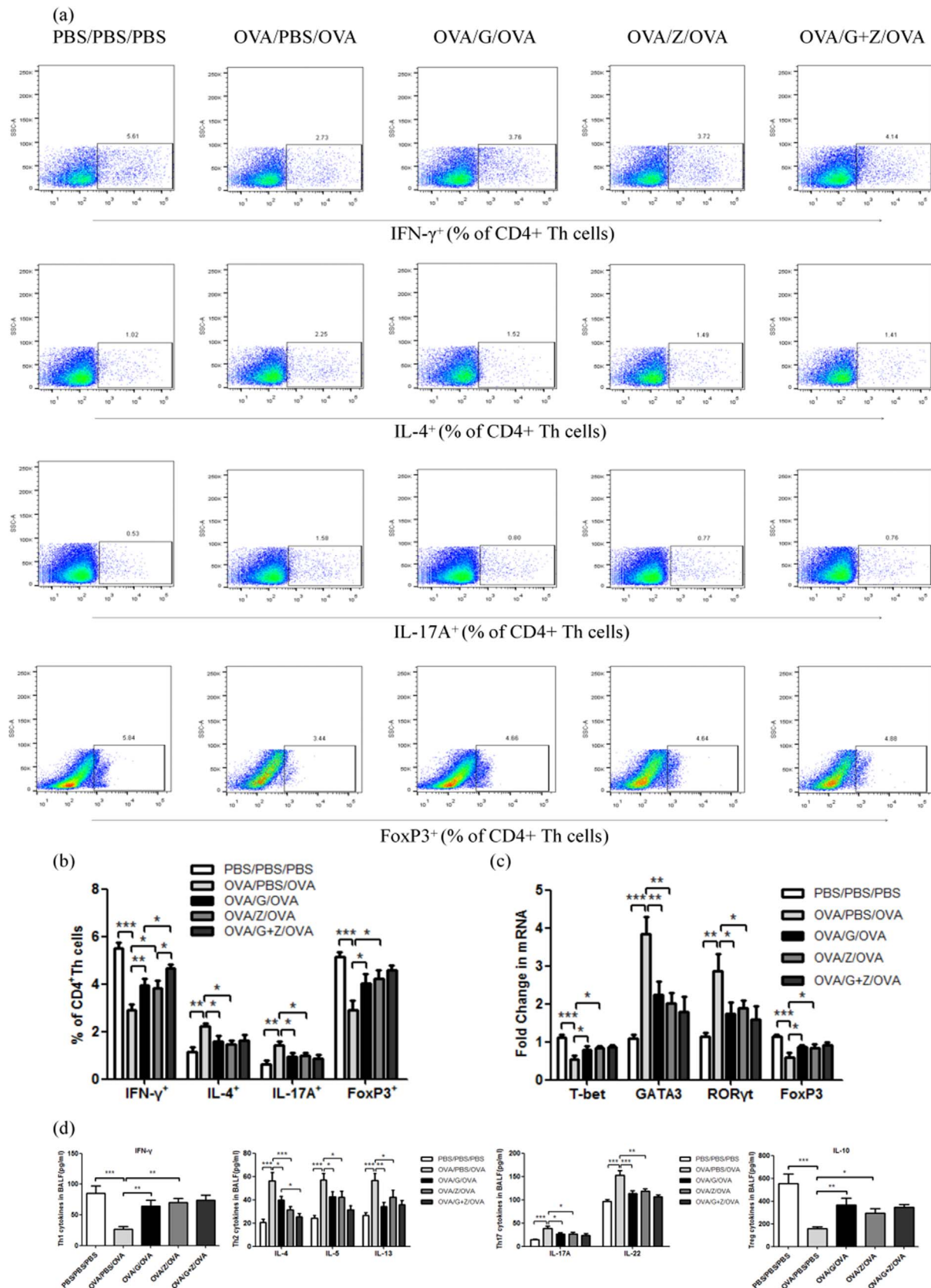


Fig. 4. Inhibited Th2, Th17 differentiation by blocking NLRP3 inflammasomes in the lung tissue of OVA-induced asthmatic mice. All groups were marked as sensitization/treatment/challenge (G = glibenclamide and Z = Z-YVAD-FMK). (a) IFN- γ^+ , IL-4 $^+$, IL-17A $^+$, and FoxP3 $^+$ Th cells were examined from CD4 $^+$ (Th) cells by flow cytometric analysis. (b) The percentage of CD4 $^+$ cells expressing IFN- γ^+ , IL-4 $^+$, IL-17A $^+$, and FoxP3 $^+$ in the left lung MNCs. (c) The transcription factors T-bet (Th1 cells), GATA3 (Th2 cells), ROR γ t (Th17 cells), and FoxP3 (Treg) were measured in the right lung. (d) Th2-associated cytokines (IL-4, IL-5, and IL-13), Th1-associated cytokines (IFN- γ), Th17-associated cytokines (IL-17A and IL-22), and Treg-associated cytokines (IL-10) were measured in BALF by ELISA. All data are represented as mean $\pm$ SEM (n = 4–6 mice per group). *P < 0.05, **P < 0.01, ***P < 0.001.

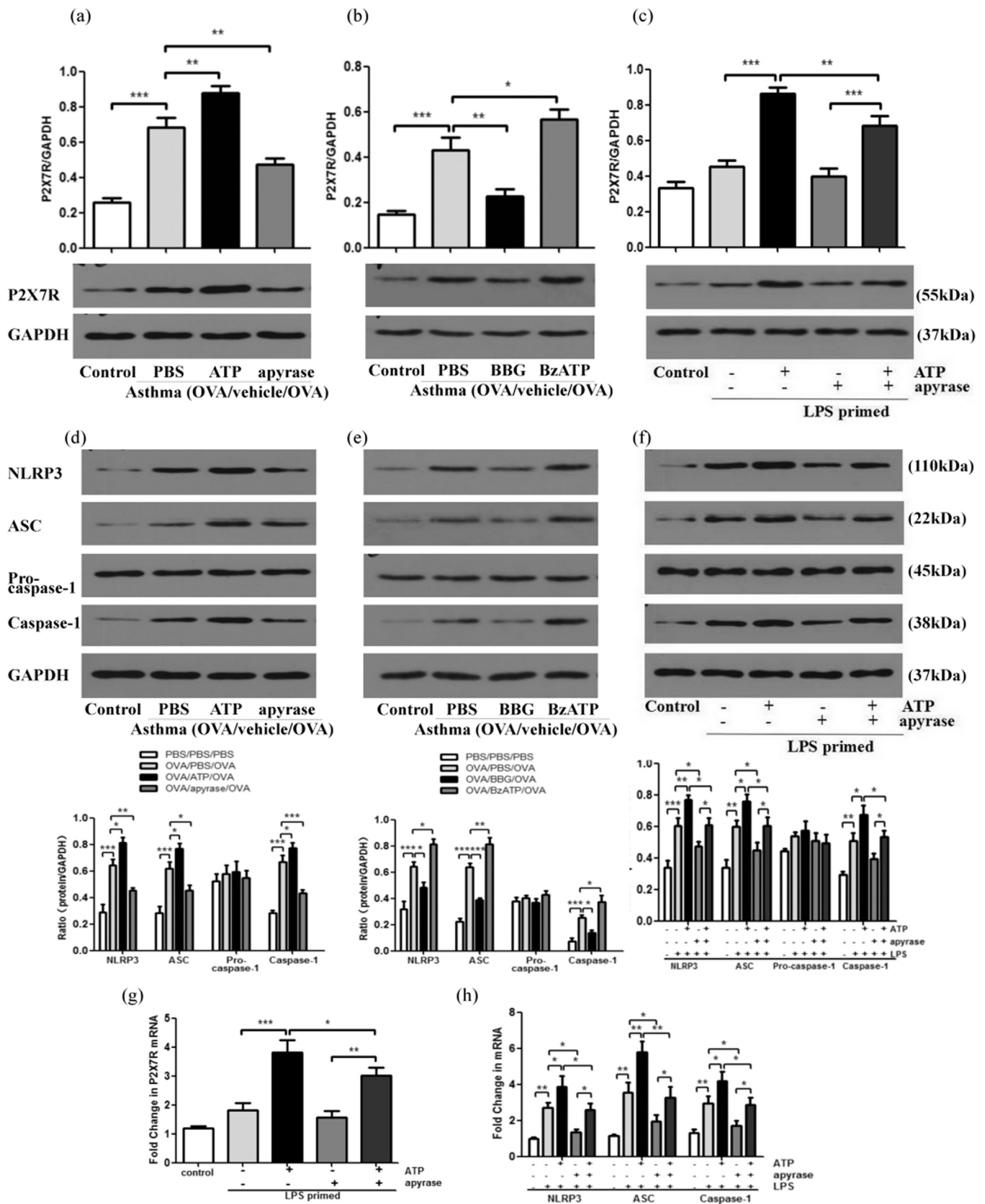


Fig. 5. Increased NLRP3 inflammasome complex expression in DCs by activating ATP/P2X7 axis. For in vivo experiment, all groups were marked as sensitization/treatment/challenge. In vitro experiment, BMDCs were primed 24 h with LPS (1 μ g/mL), and stimulated for additional 2 h with ATP (3 mM) after LPS. Apyrase (10IU/mL) was added 30 min before ATP. (a, b) Western blot analysis of P2X7 receptor and GAPDH expression in mice lung tissue. (c) Western blot and (g) real-time PCR analyses of P2X7 receptor and GAPDH expression in BMDCs. (d, e) Western blot analysis of NLRP3, ASC, pro-caspase-1, caspase-1, and GAPDH expression in mice lung tissue. (f) Western blot and (h) real-time PCR analysis of NLRP3, ASC, pro-caspase-1, caspase-1, and GAPDH expression in BMDCs. All data are represented as mean $\pm$ SEM (n = 4–6 mice per group). *P < 0.05, **P < 0.01, ***P < 0.001.

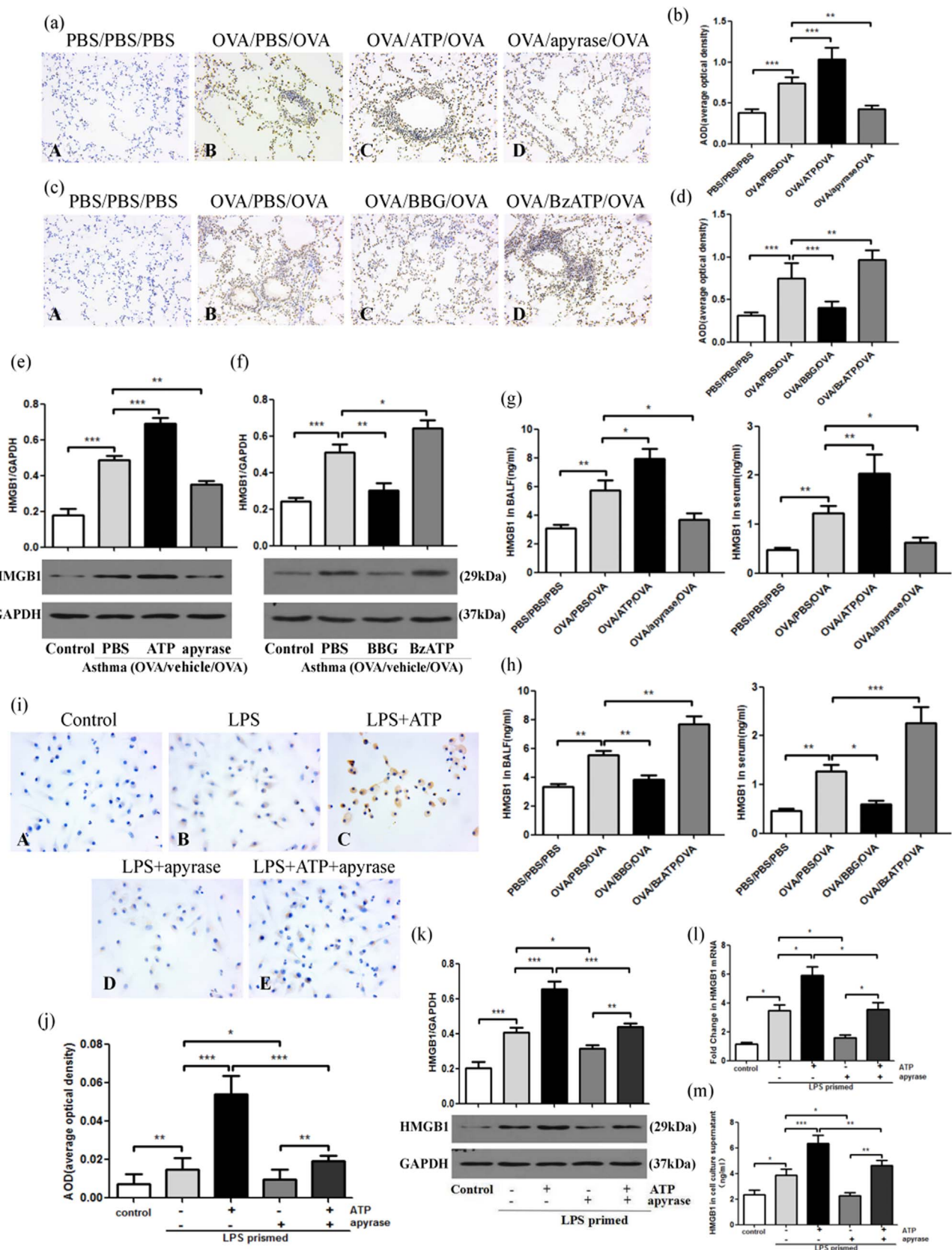


Fig. 6. Elevated expression and release of HMGB1 from DCs by activating ATP/P2X7 axis. For in vivo experiment, all groups were marked as sensitization/treatment/challenge. In vitro experiment, BMDCs were primed 24 h with LPS (1 μ g/mL), and stimulated for additional 2 h with ATP (3 mM) after LPS. Apyrase (10IU/mL) was added 30 min before ATP. (a, c) The expression of HMGB1 in mice lung tissue was detected by immunohistochemistry (200 $\times$ magnification) and (b, d) average optical density (AOD) was analyzed in different groups. (e, f) Western blot analysis of HMGB1 and GAPDH expression in mice lung tissue. (g, h) The levels of HMGB1 in serum and BALF were measured by ELISA. (i) The expression of HMGB1 in BMDCs was measured by immunohistochemistry (400 $\times$ magnification) and (j) AOD was analyzed in different groups. (k) Western blot and (l) real-time PCR analyses of HMGB1 and GAPDH expression in BMDCs. (m) The level of HMGB1 in culture supernatant of BMDCs was measured by ELISA. All data are represented as mean $\pm$ SEM (n = 4–6 mice per group). *P < 0.05, **P < 0.01, ***P < 0.001.

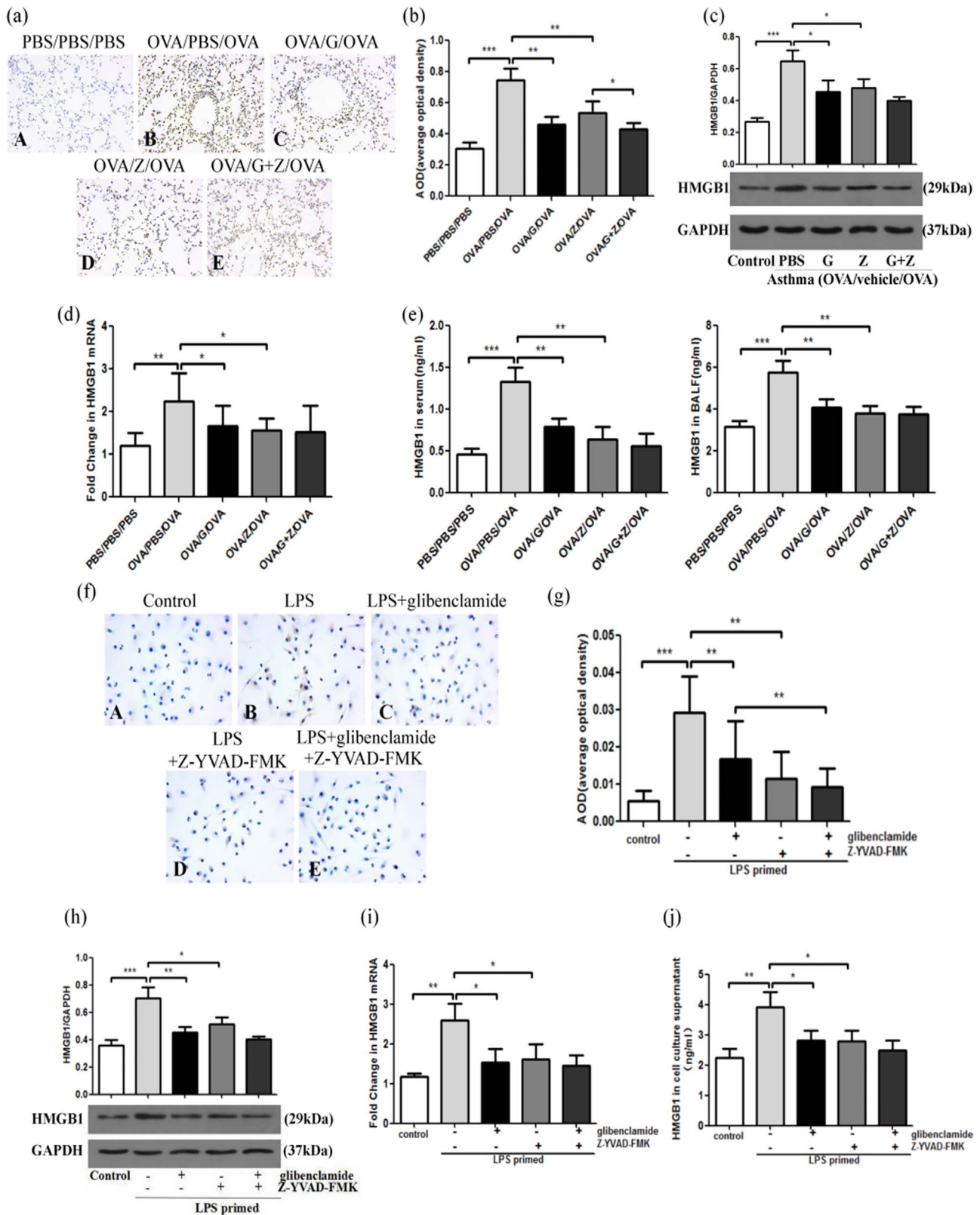


Fig. 7. Lowered expression and release of HMGB1 from DCs by blocking NLRP3 inflammasomes. For in vivo experiment, all groups were marked as sensitization/treatment/challenge (G = glibenclamide and Z = Z-YVAD-FMK). (a) The expression of HMGB1 in mice lung tissue was detected by immunohistochemistry (200× magnification) and (b) average optical density (AOD) was analyzed in different groups. (c) Western blot and (d) real-time PCR analyses of HMGB1 and GAPDH expression in mice lung tissue. (e) The levels of HMGB1 in serum and BALF were measured by ELISA. (f) The expression of HMGB1 in BMDCs was measured by immunohistochemistry (400× magnification) and (g) AOD was analyzed in different groups. (h) Western blot and (i) real-time PCR analyses of HMGB1 and GAPDH expression in BMDCs. (j) The level of HMGB1 in culture supernatant of BMDCs was measured by ELISA. All data are represented as mean $\pm$ SEM (n = 4–6 mice per group). *P < 0.05, **P < 0.01, ***P < 0.001.

hand, may result in the reduction in the expression and release of HMGB1.

3.7. The inhibition of NLRP3 inflammasome reduced HMGB1 expression and release in DCs

Next, we tested whether NLRP3 inflammasome may influence HMGB1 expression and release from DCs and if ATP/P2X7 axis induced HMGB1 expression and release by activating NLRP3 inflammasome. Immunohistochemical analysis of the lung tissue showed that the number of HMGB1-positive cells were significantly lower in glibenclamide and Z-YVAD-FMK treatment groups as compared with the asthma group (Fig. 7a, b). Furthermore, the expression of HMGB1 was significantly lower in lung tissues of glibenclamide and Z-YVAD-FMK treatment group as compared with PBS-treated group by western blot (Fig. 7c) and RT-PCR analyses (Fig. 7d). Similar results were observed with ELISA for the release of HMGB1 in the BALF and serum (Fig. 7e). Furthermore, In vitro experiment the expression of HMGB1 was significantly lower in BMDCs derived from glibenclamide and Z-YVAD-FMK treatment group stimulated with LPS as compared with BMDCs derived from the single LPS stimulation group (Fig. 7f, g). Glibenclamide and Z-YVAD-FMK stimulation markedly reduced the expression of HMGB1 (Fig. 7h) and its mRNA in LPS-primed BMDCs (Fig. 7i). Lower levels of HMGB1 were detected in serum and BALF from glibenclamide and Z-YVAD-FMK treatment group as compared with PBS treatment group. Glibenclamide and Z-YVAD-FMK stimulation also reduced the secretion of HMGB1 into the culture medium from LPS-primed BMDCs (Fig. 7j). Thus, in vivo and in vitro inhibition of NLRP3 inflammasome may reduce HMGB1 expression in the nucleus and cytoplasm of DCs and decrease its release into the extracellular region. In general, ATP/P2X7 axis may activate NLRP3 inflammasome to form the ATP/P2X7-NLRP3 axis, which mediates HMGB1 expression and secretion in DCs and affects its participation in allergic airway inflammation, AHR, and polarization of Th2 and Th17 cells in asthma.

4. Discussion

In our present study, we demonstrate that ATP/P2X7 axis and NLRP3 inflammasome mediate airway inflammation, mucus production in the lung tissue, AHR to methacholine, and differentiation of Th2, Th17 in OVA-sensitized and challenged asthma model. In DCs, ATP/P2X7 axis may activate NLRP3 inflammasome and enhance the expression of ASC, NLRP3, and caspase-1, thereby forming the ATP/P2X7-NLRP3 axis. In addition, ATP/P2X7 axis may induce the expression and release of HMGB1 from DCs. Opposite effects were observed following inhibition of NLRP3 inflammasome. Thus, the ATP/P2X7-NLRP3 axis may participate in the regulation of allergic airway inflammation, AHR, and polarization of Th2, Th17 cells in asthma by mediating HMGB1 expression and release from DCs.

As an endogenous danger signal, ATP is the only known physiological activator of P2X7 receptor, which is selectively expressed on macrophages and DCs. eATP and P2X7 receptor contribute to the development of inflammatory response with many inflammatory mediators [47]. ATP levels were increased in BALF from asthmatic patients and mice; neutralization of intrapulmonary ATP or inhibition of the purinergic receptor by antagonists may significantly reduce airway cardinal features of asthma, including airway inflammation and bronchial hyper-reactivity. Exogenous ATP may enhance the Th2-priming capacity of DCs in the mediastinal nodes [48]. Müller et al. [49] demonstrated the up-regulation in the expression of P2X7 receptor in the lung tissue of asthmatic mice; in addition, the allergic airway inflammation was markedly reduced in P2X7R-/- mice or WT mice administered with a selective P2X7 receptor antagonist. This is in line with the results of our study. The activation of ATP/P2X7 axis aggravated airway inflammation, mucus production in the lung tissue, and AHR to methacholine, while opposite effects were observed

following its inhibition. In allergic asthma, the imbalance in Th1/Th2 and Th17/Treg plays an important role. The polarization of Th2, Th17 cells in combination with their secreted cytokines initiate the allergic inflammatory response and AHR [1–5]. We found that the activation of ATP/P2X7 axis could induce Th2, Th17 inflammatory responses, while its inhibition induced the differentiation of naive Th cells into Th1 and Treg cells. This observation is consistent with the study of Müller and colleagues, wherein BMDCs deficient of P2X7r showed reduced capacity to induce Th2 immune response in asthma [49]. Zhang et al. [50] recently found that ATP and P2x7r expressions are significantly increased in neutrophilic asthma and that ATP signaling plays an important role in the development of airway inflammation and Th17 cell polarization.

NLRP3 inflammasome is an intracellular protein complex. ATP/P2X7 axis stimulates the assembly and self-oligomerization of inflammasome components and controls auto-activation of caspase-1. Upon activation, caspase-1 cleaves pro-IL-1 β and pro-IL-18 into active pro-inflammatory cytokines, which then initiate the downstream signaling pathways and drive inflammatory processes in the airway [51]. Evidences suggest that NLRP3 inflammasome is involved in the pathogenesis of asthma. Ritter et al. [52] demonstrated that NLRP3 inflammasome deficiency reduced the development of allergic airway inflammation and goblet cell hyperplasia and downregulated Th2 cytokine responses. The development of AHR requires inflammasome complex. The cleavage and activation of pro-caspase-1 results in the cleavage of pro-IL-1 β into active IL-1 β , which contributes to the complex signaling network for the development of AHR [53]. However, Allen et al. [54] found no significant differences in airway eosinophilia, histopathology, mucus production, and airway hyper-reactivity between wild-type and NLRP3-/- mice with either acute or chronic asthma. NLRP3 was not required for Th2 cell immunity following OVA-alum mediated allergic airway disease. Most of the studies agree with our results for NLRP3 inflammasome in OVA-induced asthma model, which established the functional significance of inflammasome activation as well as links between inflammasome regulation and other known mechanisms in asthma such as airway inflammation, mucus production in the lung tissue, IgE production, AHR to methacholine, and Th2, Th17 inflammatory responses.

ATP/P2X7 axis and NLRP3 inflammasome complex are expressed in macrophages, DCs, and other immune cells. DCs are considered to play major roles during the induction of T cell immune responses in allergic asthma. The maturation and activation of DCs, represented by the surface expression of major histocompatibility complex and diverse costimulatory molecules as well as the production of pro-inflammatory cytokines due to their antigen-presentation capacity, will drive subsequent Th2, Th17 immune responses [34,35]. We used in vivo and in vitro experiments to analyze their associations. In DCs, the production, cleavage, and secretion of IL-1 β and IL-18 require two signaling pathways. LPS binds to the toll-like receptor (TLR) and triggers the synthesis of pro-IL-1 β and pro-IL-18, while ATP promotes the assembly of NLRP3 inflammasome complex via P2X7 receptor by mediating caspase-1 activation from its precursor pro-caspase-1, pro-IL-18 and pro-IL-1 β cleavage into its active form, and secretion of IL-1 β and IL-18 from DCs [45,46,48]. In vitro, LPS-primed BMDCs triggered the synthesis of pro-IL-1 β and pro-IL-18. In DCs, ATP/P2X7 axis could activate NLRP3 inflammasome and augment their mRNA and protein expressions. Thus, ATP/P2X7 and NLRP3 inflammasome complex form the ATP/P2X7-NLRP3 axis in DCs that promotes the differentiation of Th2 and Th17 cells for their participation in the pathogenesis of asthma.

Our results suggest that ATP/P2X7 axis in DCs may induce HMGB1 expression and release. In general, HMGB1 acts as a key molecule of innate immune response and is secreted from the nuclear to the extracellular region during inflammation and infection [6,55]. As mentioned before, HMGB1 can be actively released from inflammatory cells, including DCs, whereas it is passively released from necrotic/apoptotic or damaged cells [6,8,10,12]. Extracellular HMGB1 is a very

“sticky” molecule and acts as an important inflammatory factor. It may interact with cell surface molecules and membrane receptors and plays biological effects only after binding to cell membrane receptors and triggering intracellular signal transduction [6,55–57]. Multiple HMGB1 receptors have been discovered. Studies have shown that cell surface RAGE has a high affinity for HMGB1 and acts as the major receptor for HMGB1. TLR2 and TLR4 are also involved in the intracellular signaling of HMGB1. HMGB1 combines with RAGE to directly affect the expression and release of inflammatory cytokines by influencing the activity of the transcription factor nuclear factor kappa B (NF- κ B); however, it may also indirectly regulate the immune response by promoting maturation and migration of immune cells. For instance, HMGB1 may promote the migration of DCs and macrophages and enhance the adhesion ability of mononuclear cells [55–57]. T helper cells and other immune cells (DCs and monocytes) express TLRs and RAGE, and HMGB1 acts on these cells and regulates their functions as an endogenous ligand of these receptors. Several studies have shown that HMGB1 levels were significantly higher in BALF and plasma from asthmatic patients and contribute to pulmonary inflammation; HMGB1 levels were correlated with the severity of asthma [13,14,58–60]. In the study of the neutrophil asthma model, recombinant HMGB1 was shown to promote the maturation of DCs and secretion of IL-23, while HMGB1-activated DCs induced differentiation of naive T cells into Th17 cells and secretion of IL-17A. Anti-HMGB1 weakened asthmatic airway inflammation and AHR, reduced the quantity of Th17 cells in the lung, and lowered cytokines levels of Th17 and DCs [16,17]. Furthermore, exogenous HMGB1 is known to aggravate eosinophilic airway inflammation of asthma, wherein a dominance of Th2-type response induced by HMGB1 plays an important role. The level of IL-17, which is generally produced by Th17 cells, was increased in the airway [18,19]. In 2015, Hou et al. [61] found that anti-HMGB1 antibody therapy reduced the level of IgE and inflammatory cytokines and decreased inflammatory cell accumulation, AHR, mucus production, and airway remodeling in chronic asthmatic mouse model. These studies show that HMGB1 participates in the regulation of airway inflammation, AHR, and airway re-modeling of allergic asthma and promotes Th2, Th17-type responses in lung. These observations in conjugation with the results of the present study suggest that ATP/P2X7 axis in DCs enhances the expression of HMGB1 and its release from the nucleus into the extracellular region, wherein it participates in the regulation of allergic airway inflammation and AHR and promotes Th2, Th17 inflammatory responses.

We show that ATP/P2X7 axis in DCs may activate NLRP3 inflammasome and enhance the expression and release of HMGB1. We suggest that ATP/P2X7 axis may further enhance the expression and release of HMGB1 by activating NLRP3 inflammasome in DCs and that HMGB1 acts downstream of the ATP/P2X7-NLRP3 axis, wherein it is regulated to promote Th2, Th17 inflammatory responses and participate in the regulation of allergic airway inflammation and AHR in asthma. We further tested if NLRP3 inflammasome may affect HMGB1 expression and release from DCs. Previous observations showed that NLRP3 inflammasome regulates IL-1 β maturation as well as HMGB1 release from some immune cells in response to pathogenic exposure. Caspase-1 is required for HMGB1 release [32,33,62–64]. Lamkanfi et al. [65] suggested that the secretion of HMGB1 from LPS-primed macrophages required NLRP3 inflammasome, while caspase-1 activation provides an important insight into the upstream mechanisms of HMGB1 release. These observations are consistent with our results. In vivo and in vitro studies revealed that the expression and secretion of HMGB1 in DCs was obviously reduced following treatment with NLRP3 and caspase-1 inhibitors, thereby confirming our hypothesis that NLRP3 inflammasome mediates HMGB1 expression and release from DCs.

In conclusion, HMGB1 is the downstream molecule of the ATP/P2X7-NLRP3 axis, which participates in the regulation of airway inflammation and AHR and promotes Th2, Th17 inflammatory responses in asthmatic mice by mediating HMGB1 expression and secretion from

DCs.

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Disclosures

The authors declare that they have no conflict of interests.

References

- [1] H.Y. Kim, R.H. DeKruyff, D.T. Umetsu, The many paths to asthma: phenotype shaped by innate and adaptive immunity, *Nat. Immunol.* 11 (2010) 577–584.
- [2] L. Dechene, TH1/TH2 immune response, *J. Allergy Clin. Immunol.* 110 (2002) 539–540.
- [3] H.Z. Shi, X.J. Qin, CD4CD25 regulatory T lymphocytes in allergy and asthma, *Allergy* 60 (2005) 986–995.
- [4] H. Jiang, X. Wu, H. Zhu, et al., FOXP3(+)Treg/Th17 cell imbalance in lung tissues of mice with asthma, *Int. J. Clin. Exp. Med.* 8 (3) (2015) 4158–4163.
- [5] L. Cosmi, F. Liotta, E. Maggi, S. Romagnani, et al., Th17 cells: new players in asthma pathogenesis, *Allergy* 66 (2011) 989–998.
- [6] M.T. Lotze, K.J. Tracey, High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal, *Nat. Rev. Immunol.* 5 (4) (2005) 331–342.
- [7] J.H. Youn, M.S. Kwak, J. Wu, et al., Identification of lipopolysaccharide-binding peptide regions within HMGB1 and their effects on subclinical endotoxemia in a mouse model, *Eur. J. Immunol.* 41 (2011) 2753–2762.
- [8] K.R. Diener, N. Al-Dasooqi, E.L. Lousberg, et al., The multifunctional alarmin HMGB1 with roles in the pathophysiology of sepsis and cancer, *Immunol. Cell Biol.* 91 (2013) 443–450.
- [9] A. Rouhiainen, S. Tumova, L. Valmu, et al., Pivotal advance: analysis of proinflammatory activity of highly purified eukaryotic recombinant HMGB1(amphotericin), *J. Leukoc. Biol.* 81 (2007) 49–58.
- [10] R. Saenz, D. Futral, L. Leutenetz, et al., TLR4-dependent activation of dendritic cells by an HMGB1-derived peptide adjuvant, *J. Transl. Med.* 12 (2014) 211.
- [11] D. Yang, Q. Chen, H. Yang, et al., High mobility group box-1 protein induces the migration and activation of human dendritic cells and acts as an alarmin, *J. Leukoc. Biol.* 81 (1) (2007) 59–66.
- [12] D. Messmer, H. Yang, G. Telusma, et al., High mobility group box protein 1: an endogenous signal for dendritic cell maturation and Th1 polarization, *J. Immunol.* 173 (1) (2004) 307–313.
- [13] T. Watanabe, K. Asai, H. Fujimoto, et al., Increased levels of HMGB-1 and endogenous secretory RAGE in induced sputum from asthmatic patients, *J. Respir. Med.* 105 (2011) 519–525.
- [14] E.J. Shim, E. Chun, H.S. Lee, et al., The role of high-mobility group box-1 (HMGB1) in the pathogenesis of asthma, *Clin. Exp. Allergy* 42 (2012) 958–965.
- [15] C.C. Lee, Y.T. Lai, H.T. Chang, et al., Inhibition of high-mobility group box 1 in lung reduced airway inflammation and remodeling in a mouse model of chronic asthma, *Biochem Pharmacol.* 86 (2013) 940–949.
- [16] F. Zhang, G. Huang, B. Hu, et al., Anti-HMGB1 neutralizing antibody ameliorates neutrophilic airway inflammation by suppressing dendritic cell-mediated Th17 polarization, *Mediat. Inflamm.* 2014 (2014) 257930.
- [17] F. Zhang, G. Huang, B. Hu, et al., Recombinant HMGB1 A box protein inhibits Th17 responses in mice with neutrophilic asthma by suppressing dendritic cell-mediated Th17 polarization, *Int. Immunopharmacol.* 24 (2015) 110–118.
- [18] E.J. Shim, E. Chun, H.S. Lee, et al., Eosinophils modulate CD4(+) T Cell responses via high mobility group box-1 in the pathogenesis of asthma, *Allergy Asthma Immunol. Res.* 7 (2015) 190–194.
- [19] L. Ma, J. Zeng, B. Mo, et al., High mobility group box 1: a novel mediator of Th2-type response-induced airway inflammation of acute allergic asthma, *J. Thorac. Dis.* 7 (2015) 1732–1741.
- [20] C. Hou, J. Kong, Y. Liang, et al., HMGB1 contributes to allergen-induced airway remodeling in a murine model of chronic asthma by modulating airway inflammation and activating lung fibroblasts, *Cell Mol. Immunol.* 12 (2015) 409–423.
- [21] F. Di Virgilio, D. Dal Ben, A.C. Sarti, et al., The P2X7 receptor in infection and inflammation, *Immunology* 47 (1) (2017) 15–31.
- [22] R.R. Hodges, J. Vrovljanis, R. Scott, Identification of P2X3 and P2X7 Purinergic Receptors Activated by ATP in Rat Lacrima Gland, *Invest. Ophthalmol. Vis. Sci.* 52 (6) (2011) 3254–3263.
- [23] E.I. Elliott, F.S. Sutterwala, Initiation and perpetuation of NLRP3 inflammasome activation and assembly, *Immunol. Rev.* 265 (1) (2015) 35–52.
- [24] A.G. Besnard, N. Guillou, J. Tschopp, et al., NLRP3 inflammasome is required in murine asthma in the absence of aluminum adjuvant, *Allergy* 66 (2011)

- 1047–1057.
- [25] M. Kool, V. Pétrilli, T. De Smedt, et al., Cutting edge: alum adjuvant stimulates inflammatory dendritic cells through activation of the NALP3 inflammasome, *J. Immunol.* 181 (2008) 3755–3759.
 - [26] S.R. Kim, D.I. Kim, S.H. Kim, et al., NLRP3 inflammasome activation by mitochondrial ROS in bronchial epithelial cells is required for allergic inflammation, *Cell Death Dis.* 5 (10) (2014) e1498.
 - [27] M. Bruchard, C. Rebé, V. Derangère, et al., The receptor NLRP3 is a transcriptional regulator of TH2 differentiation, *Nat. Immunol.* 16 (8) (2015) 859–870.
 - [28] M. Sawada, T. Kawayama, H. Imaoka, et al., IL-18 induces airway hyperresponsiveness and pulmonary inflammation via CD4⁺ T cell and IL-13, *PLoS One* 8 (1) (2013) e54623.
 - [29] L. Vande Walle, T.D. Kanneganti, M. Lamkanfi, HMGB1 release by inflammasomes, *Virulence* 2 (2) (2011) 162–165.
 - [30] B. Lu, H. Wang, U. Andersson, et al., Regulation of HMGB1 release by inflammasomes, *Protein Cell.* 4 (3) (2013) 163–167.
 - [31] M. Lamkanfi, A. Sarkar, L. Vande Walle, et al., Inflammasome-dependent release of the alarmin HMGB1 in endotoxemia, *J. Immunol.* 185 (2010) 4385–4392.
 - [32] F.Q. Bui, L. Johnson, J. Roberts, et al., *Fusobacterium nucleatum* infection of gingival epithelial cells leads to NLRP3 inflammasome-dependent secretion of IL-1 β and the danger signals ASC and HMGB1, *Cell Microbiol.* 18 (7) (2016) 970–981.
 - [33] S.B. Willingham, I.C. Allen, D.T. Bergstralh, et al., NLRP3 (NALP3, Cryopyrin) facilitates in vivo caspase-1 activation, necrosis, and HMGB1 release via inflammasome-dependent and -independent pathways, *J. Immunol.* 183 (2009) 2008–2015.
 - [34] M.B. Lutz, Induction of CD4⁺ regulatory and polarized effector/helper T cells by dendritic cells, *Immune Netw.* 6 (2016) 13–25.
 - [35] H. Vroman, B. van den Blink, M. Kool, Mode of dendritic cell activation: the decisive hand in Th2/Th17 cell differentiation. Implications in asthma severity? *Immunobiology* 220 (2015) 254–261.
 - [36] P. Li, J. Cao, Y. Chen, et al., Apyrase protects against allergic airway inflammation by decreasing the chemotactic migration of dendritic cells in mice, *Int. J. Mol. Med.* 34 (1) (2014) 269–275.
 - [37] P. Li, Y. Gao, J. Cao, et al., CD39⁺ regulatory T cells attenuate allergic airway inflammation, *Clin. Exp. Allergy* 45 (6) (2015) 1126–1137.
 - [38] W. Cui, S. Zhang, Z. Cai, et al., The antidiabetic agent glibenclamide protects airway hyperresponsiveness and inflammation in mice, *Inflammation* 38 (2) (2015) 835–845.
 - [39] M. Lamkanfi, J.L. Mueller, A.C. Vitari, et al., Glyburide inhibits the Cryopyrin/Nalp3 inflammasome, *J. Cell Biol.* 187 (1) (2009) 61–70.
 - [40] S.S. Kang, M.P. Keasey, T. Hagg, P2X7 receptor inhibition increases CNTF in the subventricular zone, but not neurogenesis or neuroprotection after stroke in adult mice, *Transl. Stroke Res.* 4 (5) (2013) 533–545.
 - [41] S. Chen, Q. Ma, P.R. Krafft, et al., P2X7R/cryopyrin inflammasome axis inhibition reduces neuroinflammation after SAH, *Neurobiol. Dis.* 58 (2013) 296–307.
 - [42] C. Csölle, M. Baranyi, G. Zsilla, et al., Neurochemical changes in the mouse hippocampus underlying the antidepressant effect of genetic deletion of P2X7 receptors, *PLoS One* 8 (6) (2013) e66547.
 - [43] T. Kølsgaard, C. Enevold, K. Bendtsen, et al., Cholesterol crystals enhance TLR2- and TLR4-mediated pro-inflammatory cytokine responses of monocytes to the proatherogenic oral bacterium *Porphyromonas gingivalis*, *PLoS One* 12 (2) (2017) e0172773.
 - [44] T.H. Chang, J.H. Huang, H.C. Lin, et al., Dectin-2 is a primary receptor for NLRP3 inflammasome activation in dendritic cell response to *Histoplasma capsulatum*, *PLoS Pathog.* 13 (7) (2017) e1006485.
 - [45] M. Karmakar, M.A. Katsnelson, G.R. Dubyak, et al., Neutrophil P2X7 receptors mediate NLRP3 inflammasome-dependent IL-1 β secretion in response to ATP, *Nat. Commun.* 15 (7) (2016) 10555.
 - [46] Q. Xie, W. Shen, J. Zhong, et al., Lipopolysaccharide/adenosine triphosphate induces IL-1 β and IL-18 secretion through the NLRP3 inflammasome in RAW264.7 murine macrophage cells, *Int. J. Mol. Med.* 34 (1) (2014) 341–349.
 - [47] J.M. Teixeira, M.C.G. De Oliveira-Fusaro, C.A. Parada, et al., Peripheral P2X7 receptor-induced mechanical hyperalgesia is mediated by bradykinin, *Neuroscience* 26 (277) (2014) 163–173.
 - [48] M. Idzko, H. Hammad, M. van Nimwegen, et al., Extracellular ATP triggers and maintains asthmatic airway inflammation by activating dendritic cells, *Nat. Med.* 13 (2007) 913–919.
 - [49] T. Müller, R.P. Vieira, M. Grimm, et al., A potential role for P2X7R in allergic airway inflammation in mice and humans, *Am. J. Respir. Cell Mol. Biol.* 44 (4) (2011) 456–464.
 - [50] F. Zhang, X. Su, G. Huang, et al., Adenosine triphosphate promotes allergen-induced airway inflammation and Th17 cell polarization in neutrophilic asthma, *J. Immunol. Res.* 2017 (2017) 5358647.
 - [51] H. Im, A.J. Ammit, The NLRP3 inflammasome: role in airway inflammation, *Clin. Exp. Allergy* 44 (2) (2014) 160–172.
 - [52] M. Ritter, K. Straubinger, S. Schmidt, et al., Functional relevance of NLRP3 inflammasome-mediated interleukin (IL)-1 β during acute allergic airway inflammation, *Clin. Exp. Immunol.* 178 (2) (2014) 212–223.
 - [53] F. Feng, Z. Li, E.N. Potts-Kant, et al., Hyaluronan activation of the Nlrp3 inflammasome contributes to the development of airway hyperresponsiveness, *Environ. Health Perspect.* 120 (12) (2012) 1692–1698.
 - [54] I.C. Allen, C.M. Jania, J.E. Wilson, et al., Analysis of NLRP3 in the development of allergic airway disease in mice, *J. Immunol.* 188 (6) (2012) 2884–2893.
 - [55] J.A. Noqueira-Machado, C.M. de Oliveira Volpe, HMGB-1 as a target for inflammation controlling, *Recent Pat. Endocr. Metab. Immune Drug Discov.* 6 (3) (2012) 201–209.
 - [56] G.P. Sims, D.C. Rowe, S.T. Rietdijk, et al., HMGB1 and RAGE in inflammation and cancer, *Annu. Rev. Immunol.* 28 (2010) 367–388.
 - [57] M.A. Van Zoelen, H. Yang, S. Florquin, et al., Role of Toll-like receptor 2 and 4, and the receptor for advanced glycation end products in high-mobility group box 1 induced inflammation in vivo, *Shock* 31 (3) (2009) 280–284.
 - [58] C. Hou, H. Zhao, L. Liu, et al., High mobility group protein B1 (HMGB1) in asthma: comparison of patients with chronic obstructive pulmonary disease and healthy controls, *Mol. Med.* 17 (2011) 807–815.
 - [59] M.B. Sukkar, L.G. Wood, M. Tooze, et al., Soluble RAGE is deficient in neutrophilic asthma and COPD, *Eur. Respir. J.* 39 (2012) 721–729.
 - [60] Y. Zhou, Y.Q. Jiang, W.X. Wang, et al., HMGB1 and RAGE levels in induced sputum correlate with asthma severity and neutrophil percentage, *Hum. Immunol.* 73 (2012) 1171–1174.
 - [61] C. Hou, J. Kong, Y. Liang, et al., HMGB1 contributes to allergen-induced airway remodeling in a murine model of chronic asthma by modulating airway inflammation and activating lung fibroblasts, *Cell Mol. Immunol.* 12 (2015) 409–423.
 - [62] P.A. Keyel, How is inflammation initiated? Individual influences of IL-1, IL-18 and HMGB1, *Cytokine* 69 (1) (2014) 136–145.
 - [63] B. Lu, T. Nakamura, K. Inouye, et al., Novel role of PKR in inflammasome activation and HMGB1 release, *Nature* 488 (2012) 670–674.
 - [64] B. Lu, H. Wang, U. Andersson, et al., Regulation of HMGB1 release by inflammasomes, *Protein Cell.* 4 (3) (2013) 163–167.
 - [65] M. Lamkanfi, A. Sarkar, L.V. Walle, et al., Inflammasome-dependent release of the alarmin HMGB1 in endotoxemia, *J. Immunol.* 185 (2010) 4385–4392.