A Potential Role for $P2X_7R$ in Allergic Airway Inflammation in Mice and Humans

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$P2X_7R$ deficiency is associated with a less severe outcome in acute and chronic inflammatory disorders. Recently, we demonstrated that extracellular adenosine triphosphate is involved in the pathogenesis of asthma by modulating the function of dendritic cells (DCs). However, the role of the purinergic receptor subtype $P2X_7$ is unknown. To elucidate the role of $P2X_7R$ in allergic airway inflammation (AAI) *in vitro* and *in vivo*, $P2X_7R$ expression was measured in lung tissue and immune cells of mice or in humans with allergic asthma. By using a specific $P2X_7R$-antagonist and $P2X_7R$-deficient animals, the role of this receptor in acute and chronic experimental asthma was explored. $P2X_7R$ was found to be up-regulated during acute and chronic asthmatic airway inflammation in mice and humans. *In vivo* experiments revealed the functional relevance of this finding because selective $P2X_7R$ inhibition or $P2X_7R$ deficiency was associated with reduced features of acute and chronic asthma in the ovalbumin-alum or HDM model of AAI. Experiments with bone marrow chimeras emphasized that $P2X_7R$ expression on hematopoietic cells is responsible for the proasthmatic effects of $P2X_7R$ signaling. In the DC-driven model of AAI, $P2X_7R$-deficient DCs showed a reduced capacity to induce Th2 immunity *in vivo*. Up-regulation of $P2X_7R$ on BAL macrophages and blood eosinophils could be observed in patients with chronic asthma. Our data suggest that targeting $P2X_7R$ on hematopoietic cells (e.g., DCs or eosinophils) might be a new therapeutic option for the treatment of asthma.

Keywords: asthma; $P2X_7$; ATP; dendritic cells; eosinophils

Asthma is a chronic inflammatory airway disease clinically characterized by variable airway obstruction, excessive mucus production, and hyperresponsiveness to nonspecific stimuli. The nonresolved chronic inflammation is orchestrated by eosinophils, mast cells, Th2 lymphocytes, and dendritic cells (DCs), resulting in airway remodelling (1). Compelling recent evidence points to an important role for myeloid DCs in initiating and maintaining allergic airway inflammation (2–6).

Adenosine triphosphate (ATP) has been implicated as an important mediator and "danger signal" in acute and chronic inflammation and can be released in large amounts from various cell types after cellular stress or tissue injury (4, 7–10). Extracellular ATP and other nucleotides (e.g., ADP, UTP, and UDP) exert their effects via binding to purinergic P2-receptors, which can be subdivided into two families: (*1*) the G-protein–coupled P2YR ($P2Y_{1–14}$) and (*2*) the ligand-gated ion channels P2XR ($P2X_{1–7}$). Among the P2XR subtypes, $P2X_7R$ is particularly highly expressed in immune cells (11–13), and its behavior is unique in that, upon repetitive or prolonged exposure to high concentrations of ATP, it can form a large membrane pore permeable to hydrophilic solutes of molecular mass up to 900 kD (14).

Activation of the $P2X_7R$ on immune cells, including dendritic cells, macrophages, and neutrophils, has been involved in various proinflammatory cell responses, such as the release of reactive oxygen species, matrix metalloproteinase-9, and cytokines (IL-2, IL-6, and IL-18) and via linking to the NALP3-inflammasom pathway in production and release of mature IL-1β (14–16), which has been shown to play an important role in asthma and other chronic diseases (17). Several studies demonstrated that the absence or blocking of $P2X_7Rs$ are associated with less severe outcomes in chronic inflammatory diseases and neuropathic pain (9, 13, 18–21). Recent evidence suggests that uric acid, another danger signal also activating the NALP3-inflammasome pathway, plays a role in the induction and maintenance of allergic diseases (22, 23).

Recently we demonstrated that BALF ATP levels are increased after allergen challenge in humans and mice and that neutralizing intrapulmonary ATP levels or the application of unselective purinergic receptor antagonists can abrogate all cardinal features of experimental asthma in mice (4). However, studies exploring the contribution of $P2X_7Rs$ to the pathophysiology of allergic airway inflammation *in vivo* are lacking.

The aim of the current study was to investigate the role of $P2X_7R$ signaling in the pathogenesis of allergic airway inflammation: $P2X_7$ receptor expression in lung tissue and immune cells of mice with acute and chronic allergic airway inflammation and in patients with asthma was measured. In addition, we explored the functional relevance of $P2X_7R$ signaling for allergic airway inflammation *in vivo*. Finally, the importance of $P2X_7R$ expression on the capacity of DCs to induce Th2 priming *in vivo* was elucidated. Our data provide evidence that selective targeting of the $P2X_7R$ on hematopoietic cells might be a new therapeutic option for the treatment of asthma.

CLINICAL RELEVANCE

Bronchial asthma is a major health burden in western society. Our data showing the involvement of $P2X_7R$ signaling in asthma pathophysiology could be the basis for a new therapeutic approach.

MATERIALS AND METHODS

Mice

Balb/c mice, ovalbumin–T-cell receptor (OVA-TCR) transgenic mice (DO11.10) on a Balb/c background and C57/Bl6 mice (6–8 wk old)

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were bred at the animal facilities at the University Hospital Freiburg. $P2X_7R$-deficient mice ($P2X_7R-/-$) on C57Bl/6 background were kindly provided by GlaxoSmithKline (London, UK) and backcrossed with our C57/Bl6 strains (24). All experiments were performed according to institutional guidelines of the animal ethics committee from the German government.

OVA-Alum Model of Allergic Airway Inflammation

For the acute model, different mice strains were sensitized to OVA (Worthington Biochemical, Lakewood, NJ) via intraperitoneal injection of OVA/alum on Days 0 and 7 and were challenged with OVA aerosols on Days 19 to 21 delivered from a jet nebulizer delivering 1% OVA in PBS for 30 minutes. Twenty-four hours after the last OVA exposure, bronchoalveolar lavage (BAL) was performed with 3×1 ml of Ca^{2+}-free and Mg^{2+}-free PBS (Invitrogen, Carlsbad, CA) supplemented with 0.1 mM sodium EDTA, followed by lung resection and storage in OCT freezing medium.

In the chronic model, female BALB/c mice were sensitized as described above and challenged with OVA aerosols 3 d/wk over 6 weeks. During the last 2 weeks, animals were treated intratracheally with vehicle or KN62 before allergen challenge.

House Dust Mite–Induced Allergic Airway Inflammation

Female BALB/c mice (6–9 wk old) were anesthetized using ketamine/xylazine and injected intratracheally with 100 μg *Dermatophagoides pteronyssinus* extracts (Greer Laboratories, Lenoir, NC) or vehicle (PBS) as a negative control on Day 0. On Days 7 and 14, animals received an intratracheal injection of house dust mite (HDM) (100 μg) or HDM admixed with KN62. Animals were assessed for the classical features of allergic airway inflammation (BAL eosinophilia, histology) on Day 17, as previously described (25).

Th2 Sensitization Induced by Intratracheal Injection of Bone Marrow–Derived DCs

DCs were prepared as previously described (3) (details are provided in the online supplement). Cells were pulsed overnight with 100 μg/ml LPS-low OVA (Worthington Biochemicals, Lakewood, NJ) or vehicle. After antigen pulsing, nonadherent DCs were collected and washed to remove free OVA. For *in vivo* experiments, C57Bl/6 mice were anesthetized on Day 0 with ketamin/xylazine, and 1×10^6 vehicle-wild type (wt)-DCs, OVA-wt-DCs, vehicle-$P2X_7R-/-$ DCs, or OVA-$P2X_7R-/-$ DCs were instilled through the vocal cords as described (3). On Days 10 through 12, mice were exposed to OVA aerosols (30 min). Mice were killed 24 hours after the last aerosol.

BAL Fluid Collection from Patients with Asthma and Healthy Control Subjects

BAL fluid (BALF) of 10 patients with mild allergic asthma and 10 healthy control subjects was collected at the University of Freiburg (*see* Table E2 in the online supplement for patient characteristics). Patients with malignancies or signs or symptoms of acute infections were excluded. In the control group, patients taking cardiac or pulmonary medications were excluded. Patients with asthma were diagnosed according to the current guidelines, and inhaled corticosteroids were withheld for 4 weeks before bronchoscopy. The study was approved by the local ethics committee of Freiburg, and all participants gave their written informed consent. BALF was collected and processed as described (26).

Blood Donors for Eosinophils

Eight patients with a diagnosis of allergic asthma were recruited on the basis of airway hyperresponsiveness, positive allergen skin-prick tests, or elevated total or specific IgE concentrations. Eight subjects without any history of allergic signs or symptoms, a negative skin prick test to a large panel of allergens, and normal IgE levels served as control subjects (Table E2). Participants gave their written informed consent. The study was approved by the local ethics committee. Inhaled corticosteroids were withdrawn at least 14 days before blood was taken. None of the patients took systemic corticosteroids.

Statistical Analysis

If not stated otherwise for most experiments, the statistical significance of differences between samples was calculated using ANOVA, followed by Bonferroni comparison test. Differences were considered significant at $P < 0.05$.

RESULTS

Up-Regulation of $P2X_7R$ Expression in Acute and Chronic Allergic Airway Inflammation

To better define the role of $P2X_7R$ signaling in asthmatic airway inflammation, we analyzed the expression of the $P2X_7R$ subtype in the lungs of mice sensitized to OVA and challenged with OVA aerosols on three consecutive days. The induction of acute allergic airway inflammation was associated with a strong up-regulation of the $P2X_7R$ subtype in lung tissue and BALF cells (Figures 1a and 1b). We also observed an up-regulation of $P2X_7R$ expression in lung tissue and BALF cells of mice after chronic allergen challenge, suggesting an important role of $P2X_7R$ in the chronic phase of asthmatic airway inflammation (Figures 1c and 1d).

Effect of Selective $P2X_7R$ Blockade on Acute and Chronic Allergic Airway Inflammation in Mice

To determine whether blocking of $P2X_7R$ during allergen challenge could influence the development of acute allergic lung

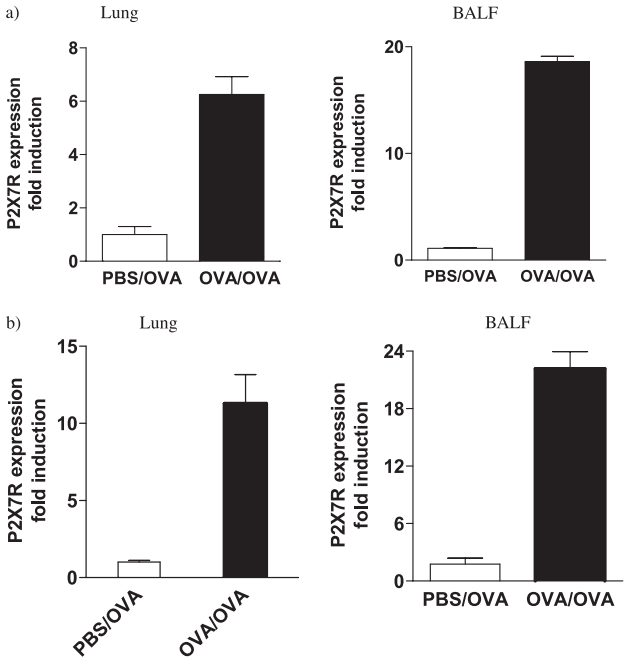

Figure 1. Up-regulation of $P2X_7R$ expression in acute and chronic allergic airway inflammation. Acute and chronic allergic airways in BALB/c animals were induced as described in Materials and Methods. After the last allergen challenge, animals were killed, bronchoalveolar lavage fluid (BALF) and whole lungs were collected, and RNA was isolated. Relative expression of $P2X_7R$ in sensitized compared with nonsensitized animals was analyzed using quantitative RT-PCR: $P2X_7R$ expression on total lung tissue of animals with (*b*) acute and (*d*) chronic allergic airway inflammation and control animals. $P2X_7R$ expression on BALF cells from animals with (*a*) acute or (*c*) chronic allergic airway inflammation and control animals. Data are shown as mean ± SEM (n = 5–8 mice per group). *$P < 0.05$. Data from one representative experiment out of three are shown. OVA = ovalbumin.

inflammation in sensitized mice, the selective $P2X_7R$ antagonist KN62 was administered intratracheally to OVA-sensitized mice before each OVA challenge. OVA-sensitized mice that received vehicle treatment before OVA aerosol challenge showed all the cardinal features of allergic airway inflammation, as seen by BALF eosinophilia, peribronchial inflammation, enhanced Th2 cytokine production by mediastinal lymph node (MLN) cells, and airway hyperresponsiveness (AHR) to metacholine compared with sham-sensitized animals (Figure 2). The intratracheal application of KN62 before each allergen challenge resulted in significantly reduced BAL-eosinophila and lymphocytosis, BAL IL-5 and IL-13 levels, peribronchial inflammation, and goblet cell hyperplasia, which was accompanied by mildly but significantly reduced IL-4, IL-5, and IL-13 production by MLN cells and attenuated AHR (Figures 2a–2e). The protective effect of KN62 was dose dependent (Table E1).

To define the role of $P2X_7R$ in the progression of airway inflammation and remodeling, a mouse model of chronic asthma was used in which sensitized mice were challenged on 3 d/wk over 6 weeks. Treatment with the selective $P2X_7R$ antagonist KN62 was performed during the last 2 weeks of the protocol. Treatment with KN62 led to a reduction in BAL-eosinophila and lymphocytosis; BALF levels of IL1-β, IL-5, IL-6, and IL-13; peribronchial inflammation; airway collagen deposition and goblet cell hyperplasia and to attenuated AHR (Figures 3a–3d).

$P2X_7R$-Deficient Mice Have Reduced Asthmatic Airway Inflammation

As proof of concept, we investigated whether $P2X_7R$ deficiency is associated with reduced allergic airway inflammation. We observed that $P2X_7R$-deficient animals ($P2X_7R-/-$) displayed a weaker (compared with wt animals treated with KN62) but significant reduction in the number of lymphocytes and eosinophils (IL-5 and IL-13) in BAL, reduced peribronchial inflammation, and reduced methacholine-induced AHR. These changes were accompanied by decreased IL-4, IL-5, and IL-13 production by restimulated MLN cells (Figures 4a–4d).

To discriminate between the role of $P2X_7R$ signaling on hematopoietic and structural cells in allergic airway inflammation, $P2X_7R-/-$ and wt bone marrow (BM) chimeras were sensitized and challenged with OVA. $P2X_7R$ deficiency of the donor ($P2X_7R-/-$ → wt) but not of the recipient (wt → $P2X_7R-/-$) led to decreased BAL eosinophila and Th2 cytokine levels in MLN cells (Figures 4e–4f).

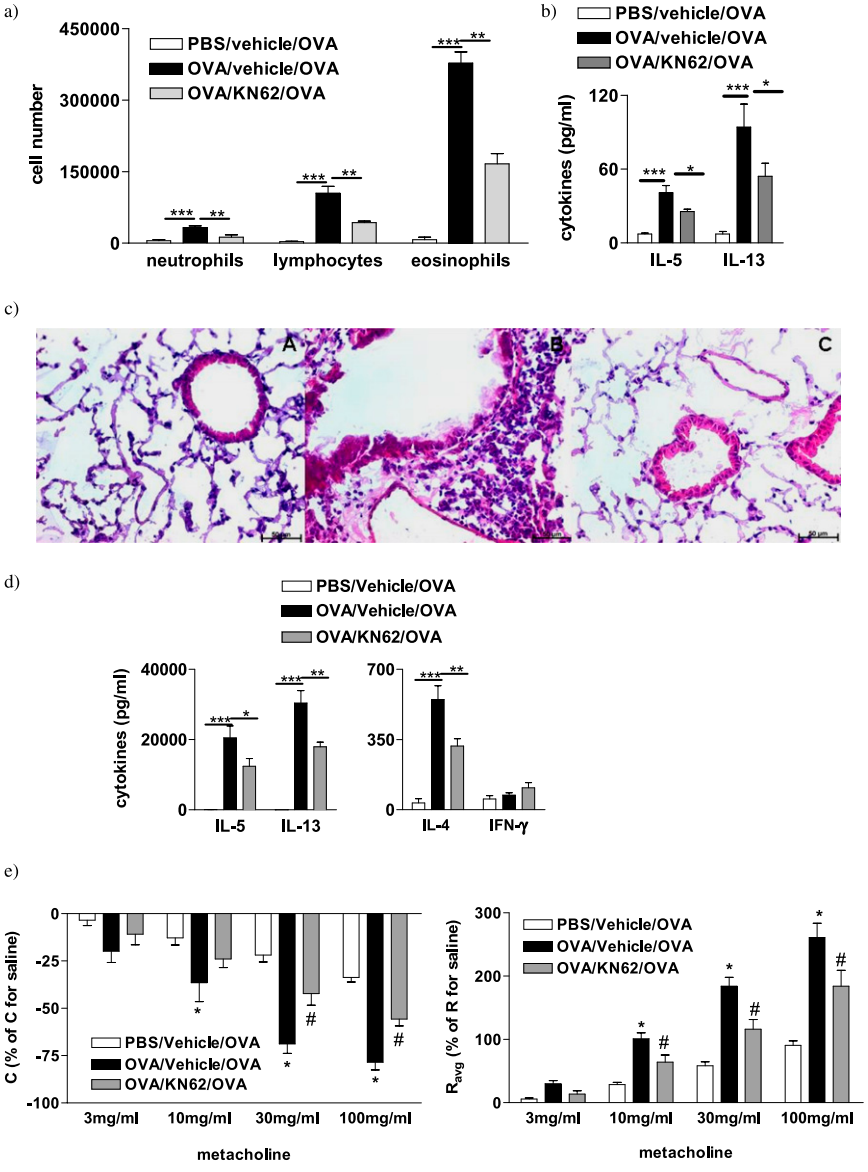

Figure 2. Intrapulmonary treatment with $P2X_7R$ antagonist KN62 suppresses acute allergic airway inflammation. Mice were sensitized by intraperitoneal injection of OVA/alum on Days 0 and 7 and were exposed on Days 19 to 21 to OVA aerosols. Before each aerosol, mice received intraperitoneal injection of 1 μM KN62. Groups are coded as sensitization, treatment, and challenge. (*a* and *b*) BALF was analyzed by flow cytometry, and BALF cytokines were measured by ELISA. (*c*) H&E staining of lung sections (*A*: PBS/vehicle/OVA; *B*: OVA/vehicle/OVA; *C*: OVA/KN62/OVA). (*d*) Cytokine production in mediastinal lymph node cells restimulated *in vitro* for 4 days with OVA. Data are mean ± SEM (n = 6–8 mice per group). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. (*e*) Bronchial hyperresponsiveness to various doses of aerosolized methacholine 24 hours after the last antigen exposure were measured by changes in average resistance (R) and lung compliance (C) in mechanically ventilated mice. Data are mean ± SEM (n = 8 mice per group). $^*P < 0.01$ PBS/OVA versus OVA/OVA; $^{\#}P < 0.05$ vehicle-treated versus KN62-treated animals. One experiment out of three is shown.

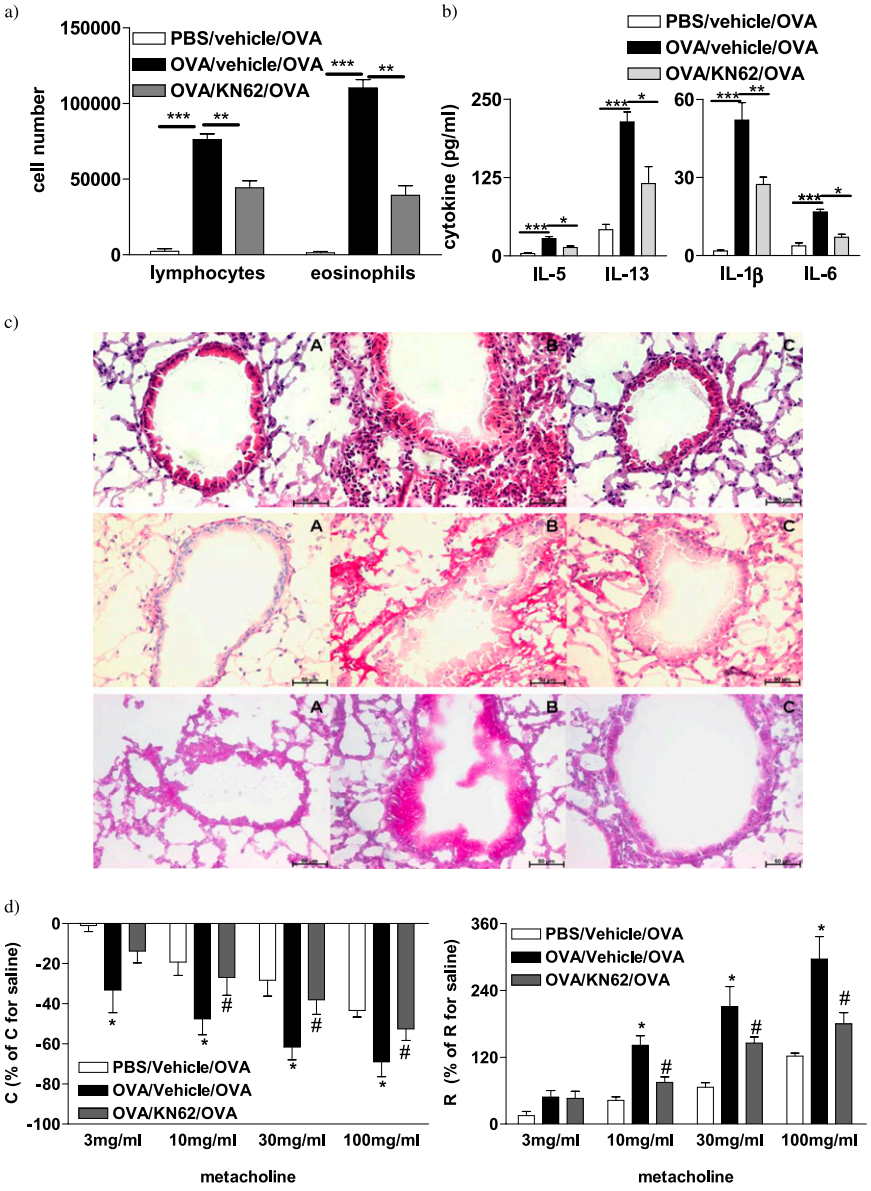

Figure 3. Effect of KN62 on chronic allergic airway remodeling. (*a* and *b*) BALF was analyzed by flow cytometry, and BALF cytokines were measured by ELISA. (*c*) H&E staining for inflammation (*upper panel*), PAS staining for goblet cell hyperplasie/mucus production (*middle panel*), and Sirius red-staining for collagen-deposition (*lower panel*) of vehicle- or KN62-treated animals with chronic allergic airway inflammation (*A*: PBS/vehicle/OVA; *B*: OVA/vehicle/OVA; *C*: OVA/KN62/OVA). Data are mean ± SEM (n = 6–8 mice per group). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. (*d*) BHR to various doses of aerosolized methacholine 24 hours after the last antigen exposure were measured by changes in average resistance (R) and lung compliance (C) in mechanically ventilated mice. Data are mean ± SEM (n = 6–8 mice per group). Data are mean ± SEM (n = 8 mice per group). *$P < 0.01$, PBS/OVA versus OVA/OVA; #$P < 0.05$, vehicle-treated versus KN62-treated animals. One experiment out of three is shown.

P2X$_7$R in HDM-Induced Allergic Airway Inflammation

The OVA-alum model has been used extensively to study asthmatic airway inflammation *in vivo*. However, to get closer to human asthma, another animal model in which eosinophilic airway inflammation was induced by intratracheal administration of HDM extract was used. Intratracheal administration of HDM extract on Days 0, 7, and 14 resulted in a strong increase in BAL eosinophils, tissue infiltration, mucus production, and Th2 cytokine levels in MLN cells. Nevertheless, the administration of the P2X$_7$R antagonist KN62 together with the allergen resulted in decreased allergic airway inflammation (Figures 5a–5c).

Dendritic Cells from P2X$_7$R−/− Migrate in Response to Extracellular ATP

Among hematopoietic cells, myeloid DCs have been shown to play an essential role in the sensitization to inhaled allergen and in the maintenance of allergic airway inflammation. Previously, we have demonstrated that ATP enhanced the recruitment of DCs to the lungs and to MLN cells. To investigate whether P2X$_7$R deficiency affects DC migration, wt and P2X$_7$R−/− animals received an intratracheal application of OVA-FITC plus vehicle, OVA-FITC plus ATPγS, or vehicle alone. Concomitant treatment with ATPγS led to an enhanced absolute number of DCs in the lungs in wt and P2X$_7$R−/− mice (Figure E1a). The number of lung MHCII+CD11c+FITC+ DCs reaching the MLNs in P2X$_7$R−/− animals was similar compared with wt animals (Figure E1b).

In vitro experiments revealed that wt and P2X$_7$R−/− DCs migrate to increasing concentrations of ATP. In accordance, bone marrow–derived DCs (BMDCs) generated from P2X$_7$R−/− animals showed the same migratory capacity to ATP compared with BMDCs generated from wt mice (Figure E1c).

Selective Inhibition of P2X$_7$R or P2X$_7$R−/− Deficiency of Bone Marrow–Derived DCs Is Associated with a Reduced Capacity To Induce Th2 Immunity *In Vitro* and *In Vivo*

It has been reported that the selective inhibition of P2X$_7$R in OVA-pulsed dendritic cells reduced their ability to stimulate IL-2 production in OVA-TCR Tg (DO11.10) T cells (27). To determine whether selective inhibition of the P2X$_7$R on DCs reduced their Th2-priming capacity *in vitro*, BMDCs of BALB/c mice were stimulated with the P2X$_7$R−/− antagonists oATP

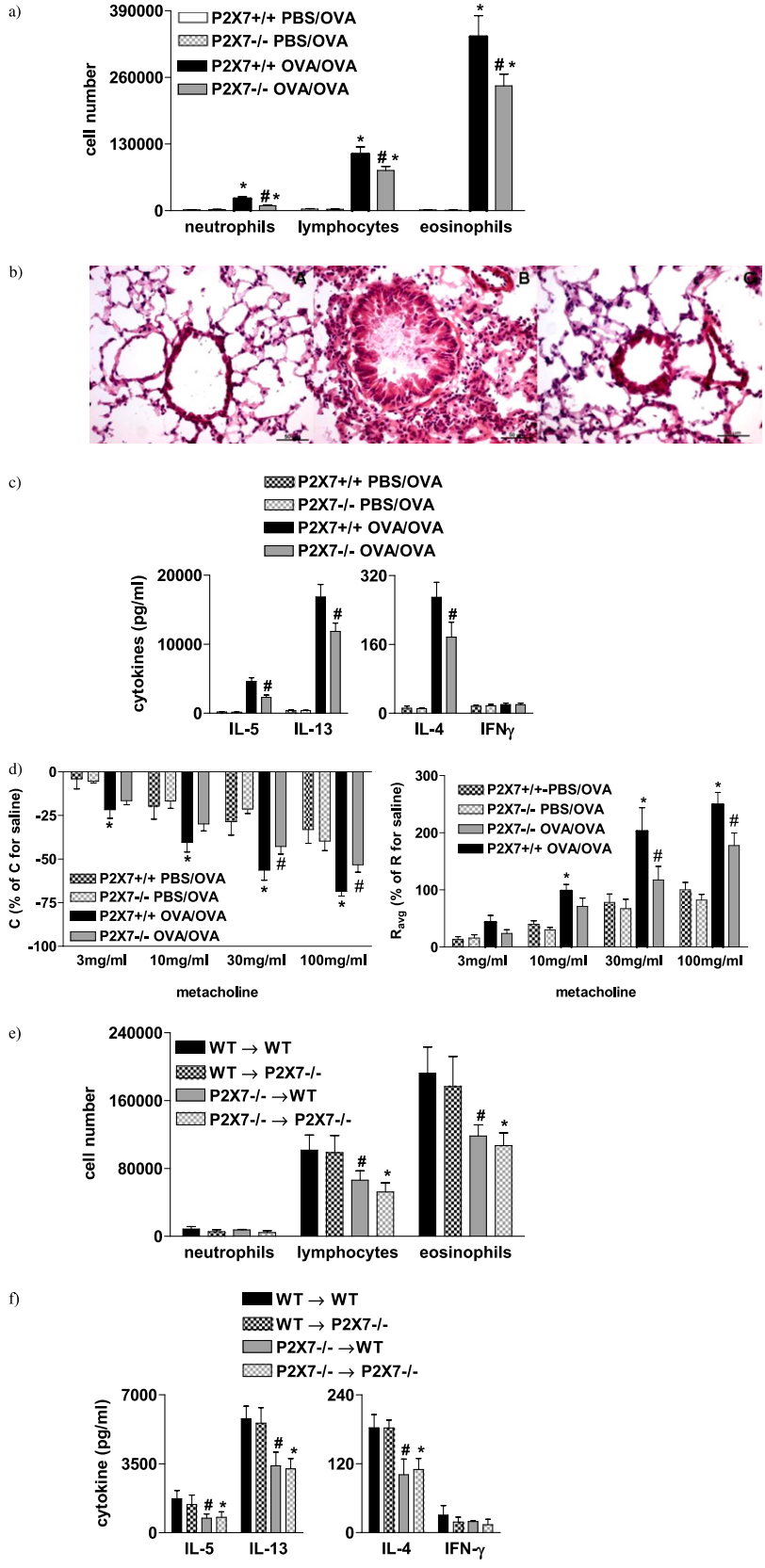

Figure 4. $P2X_7R$-deficient animals show reduced allergic airway inflammation. (*a*) BALF cell differential counts, measured by flow cytometry, of wild-type (wt) and $P2X_7R-/-$ mice sensitized by intraperitoneal injection of OVA alum at Days 0 and 7 and exposed to OVA aerosols on Days 19 through 21. (*b*) H&E staining of lung sections (*A*: $P2X_7R+/+$ PBS/OVA; *B*: $P2X_7R+/+$ OVA/OVA; *C*: $P2X_7R-/-$ OVA/OVA). (*c*) Cytokine production by mediastinal lymph node cells restimulated *in vitro* for 4 days with OVA. (*d*) Bronchial hyperresponsiveness to various doses of aerosolized methacholine 24 hours after the last antigen exposure were measured by changes in average resistance (R) and lung compliance (C) in mechanically ventilated mice. Data are mean ± SEM (n = 6–8 mice per group). *$P < 0.001$ $P2X_7R+/+$ and $P2X_7R-/-$ PBS/OVA animals versus $P2X_7R+/+$ and $P2X_7R-/-$ OVA/OVA animals; $^{\#}P < 0.05$ $P2X_7R+/+$ OVA/OVA versus $P2X_7R-/-$ OVA/OVA. Data from one representative experiment out of three experiments are shown. (*e* and *f*) $P2X_7R$ deficiency on hematopoietic system is associated with reduced allergic airway inflammation. The different bone marrow-chimeras were sensitized and challenged to OVA as described in Materials and Methods. (*e*) BALF was analyzed by flow cytometry. (*f*) Cytokine production by mediastinal lymph node cells restimulated *in vitro* for 4 days with OVA. Data are mean ± SEM (n = 5–8 mice per group). *$P < 0.05$ $P2X_7R-/-$ in $P2X_7R-/-$ versus wt in wt; $^{\#}P < 0.05$. $P2X_7R-/-$ in wt versus wt in wt. One representative experiment out of three experiments is shown.

(300 μM), KN62 (10 μM), or vehicle 30 minutes before overnight pulsing with OVA or PBS. DCs were washed twice and cocultured with purified naive CD4+ T cells from DO11.1O animals. DO11.10 cells that had been cultured with oATP-OVA-DCs or KN62-OVA-DCs for 3 or 5 days produced lower levels of IL-2 and the Th2 cytokines IL-5 and IL-13 (Figure 6a) compared with vehicle-treated DCs.

To better define the relevance of $P2X_7R$ signaling on DC function *in vivo*, we investigated whether $P2X_7R-/-$ DCs show a reduced capacity to induce Th2 immunity in a

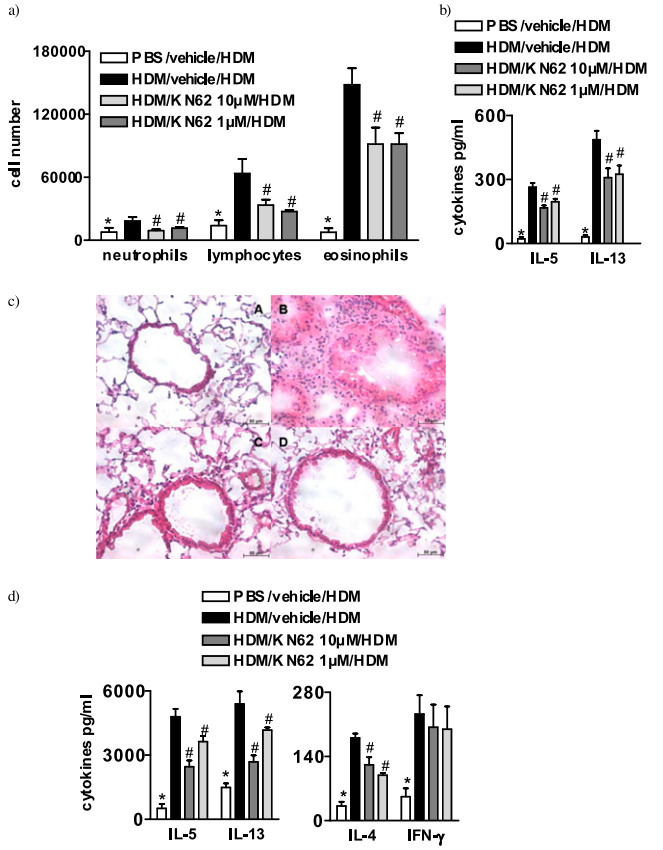

Figure 5. P2X$_7$R signaling in a house dust mite (HDM)-induced model of allergic airway inflammation. (*a* and *c*) Female BALB/c mice were exposed to HDM or to PBS (control) on Day 0. All of the mice were then challenged with HDM admixed to KN62 (10 or 1 μM) or the vehicle (PBS) on Days 7 and 14. The number of BALF cells (*a*) and levels of BALF cytokine (*b*) were determined by flow cytometry or ELISA 72 hours after challenge. (*c*) H&E staining of lung sections (*A*: PBS/vehicle/HDM; *B*: HDM/vehicle/HDM; *C*: HDM/KN62 [10 μM/HDM] and HDM/KN62 [1 μM/HDM]). (*d*) Cytokine production by MLN cells restimulated *in vitro* for 4 days with HDM. Data are means ± SEM (n = 6–8 mice per group). *$P < 0.01$ PBS/HDM versus HDM/HDM; #$P < 0.05$ HDM/vehicle/HDM versus HDM/KN62/HDM. One representative data out of three experiments is shown.

DC-driven model of allergic airway inflammation. BMDCs of wt and P2X$_7$R−/− mice were pulsed overnight with OVA or PBS and were injected intratracheally into wt animals. In animals receiving unpulsed DCs (PBS-DCs), only a few inflammatory cells were recruited to lungs after OVA challenge (Figures 6b–6d). In contrast, the intratracheal application of OVA-pulsed wt DCs was associated with a strong recruitment of lymphocytes and eosinophils into the BALF, strong peribronchial and perivascular inflammation, and production of the Th2 cytokines IL-4, IL-5, and IL-13 by MLN cells. In animals receiving OVA-pulsed P2X$_7$R−/− DCs, the classical features of allergic airway inflammation were significantly reduced (Figures 6b–6d). However, when wt OVA-DCs were injected into wt or P2X$_7$R−/− animals, no significant differences could be observed (Figure 6e). The reduced capacity of the P2X$_7$R−/− DCs might be due to a defect in OVA-induced maturation. However, P2X$_7$R deficiency did not alter OVA- or ATP-induced up-regulation of CD40, CD80, CD83, and CD86 by DCs (data not shown).

P2X$_7$-Deficient BMDCs Fail to Secrete Mature IL-1β

To address the functional consequences of P2X$_7$R deficiency in DCs, wt and P2X$_7$R−/− BMDCs were analyzed for the secretion of mature IL-1β (Figure E2) upon stimulation with LPS, OVA, and ATP *in vitro*. Only wt BMDCs released IL-1β upon stimulation with OVA, with LPS alone, or in combination with ATP.

P2X$_7$R Is Up-Regulated on BALF Leukocytes in Patients with Chronic Asthma Compared with Healthy Control Subjects

To confirm the relevance of P2X$_7$R for human asthma, we analyzed P2X$_7$R expression on BALF cells from individuals with asthma and healthy control subjects. BAL macrophages from individuals with asthma expressed higher levels of P2X$_7$R compared with healthy control subjects (Figure 7a). This up-regulation is of functional relevance because BAL macrophages from individuals with asthma secreted larger amounts of IL-1β in response to the selective P2X$_7$R agonist BzATP (Figure 7b).

Up-Regulation of P2X$_7$R on Blood Eosinophils of Patients with Asthma Compared with Healthy Control Subjects

Because we could not detect differences in P2X$_7$R expression by monocyte-derived DCs, probably due to the *in vitro* culture for 5 days, we compared P2X$_7$R expression on human eosinophils, another major effector cell in the pathogenesis of asthma. We observed a significant up-regulation of P2X$_7$R expression by eosinophils derived from individuals with asthma compared with healthy control subjects (Figure 7c). This was of functional relevance because these eosinophils produced more reactive oxygen species upon stimulation with BzATP (Figure 7d).

DISCUSSION

Compelling evidence indicates a pathophysiological relevance for the purinergic receptor subtype P2X$_7$ in acute and chronic inflammatory processes (13, 15, 21, 28, 29). Additionally, targeting P2X$_7$R signaling has been shown to decrease disease severity in different animal models of inflammatory disorders (e.g., collagen-induced arthritis, renal fibrosis, spinal cord injury, or inflammatory pain), suggesting a therapeutic potential of selective P2X$_7$R antagonists (21, 30). The selective P2X$_7$ antagonist AZ9056 (Astra-Zeneca, London, UK) is reportedly under clinical trials for the treatment of chronic inflammatory bowel disease and rheumatoid arthritis (13, 28). Although we recently demonstrated that endogenous ATP accumulating in the airways of patients with asthma and sensitized mice after allergen challenge plays an important role in allergic airway inflammation, nothing was known about the involvement of P2X$_7$ receptors (4).

In the present study, we provide evidence that P2X$_7$R signaling contributes to the pathogenesis of allergic airway inflammation. P2X$_7$R expression was up-regulated in the lungs of mice with allergic lung inflammation as well as in BALF macrophages and blood eosinophils from humans with asthma. P2X$_7$R−/− animals or animals treated with a selective P2X$_7$R antagonist showed a strong reduction in all cardinal features of acute allergic airway inflammation (including airway eosinophilia, goblet cell hyperplasia, and bronchial hyperresponsiveness to methacholine). The observation that wt animals that were treated with selective P2X$_7$R antagonist showed a stronger reduction in allergic airway inflammation compared with constitutive P2X$_7$R−/− animals might be due to the recently reported unexpected higher P2X$_7$R activity on T cells of the investigated P2X$_7$R−/− mice (31).

Our findings that P2X$_7$R blocking can suppress airway remodeling in a chronic model of experimental asthma suggest that

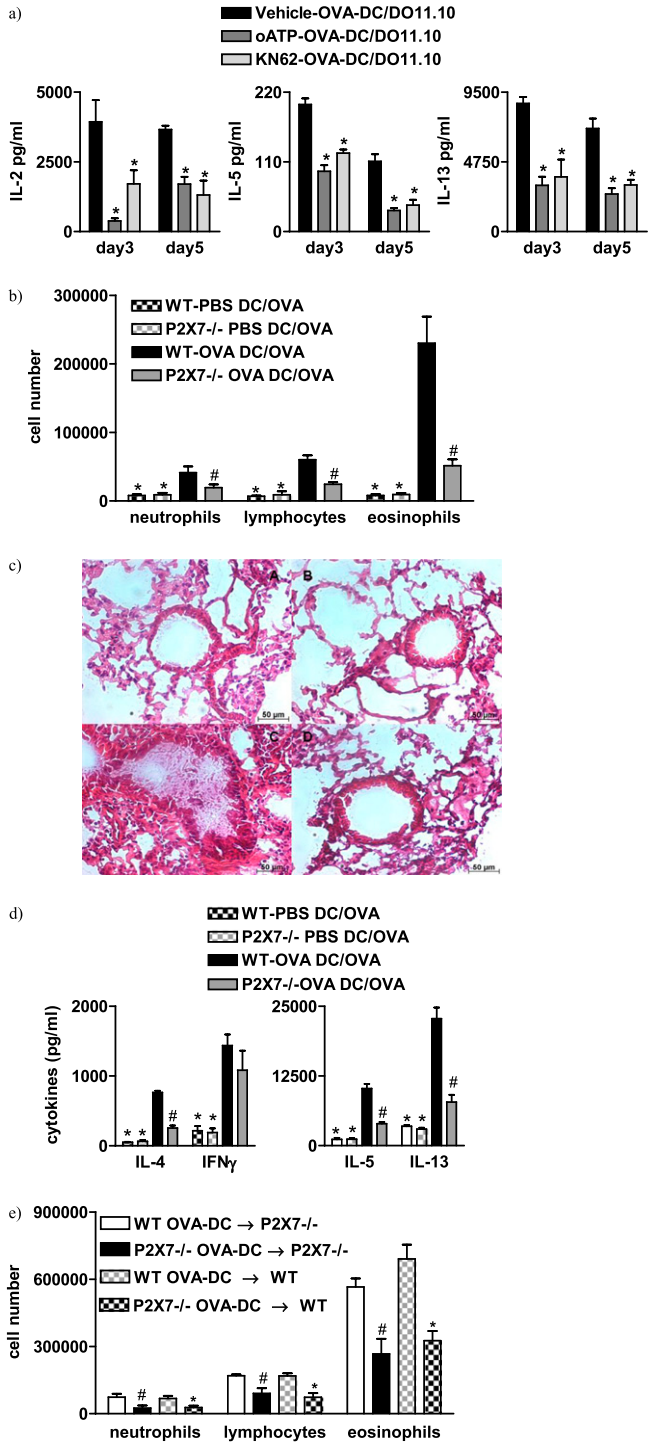

Figure 6. Involvement of functional $P2X_7R$ in T-cell priming capacity of dendritic cells (DCs) *in vitro* and *in vivo*. (*a*) OVA-DCs were incubated with different $P2X_7R$ antagonists (KN62 1 μM or oATP 300 μM) or vehicle before coculture with DO.11.10 T cells for 3 and 5 days *in vitro*. The levels of IL-2, IL-5, and IL-13 were measured in the supernants. Data are means ± SEM. $^*P < 0.01$ vehicle-OVA DC versus $P2X_7R$ antagonist-OVA-DCs. (*b–d*) On Day 0, mice received intracheal injections of WT or $P2X_7R-/-$ OVA-DCs or unpulsed WT or $P2X_7R-/-$ DCs. From Days 10 to 13, all mice were exposed to OVA aerosols. (*b*) BALF was analyzed by flow cytometry. (*c*) H&E staining of lung sections (*A*: $P2X_7R+/+$ PBS DCs/OVA; *B*: $P2X_7R-/-$ PBS/OVA; *C*: $P2X_7R+/+$ DCs OVA/OVA; *D*: $P2X_7R-/-$ DCs OVA/OVA). (*d*) Cytokine production by MLN cells restimulated *in vitro* for 4 days with OVA. Data are mean ± SEM (n = 6–8 mice per group). $^*P < 0.01$ PBS-DCs versus OVA-DCs; $^{\#}P < 0.01$ WT-OVA-DCs versus $P2X_7R-/-$ OVA-DCs. Representative data from three experiments are shown. (*e*) OVA-DCs from WT or $P2X_7R-/-$ animals were injected intratracheally in WT or $P2X_7R-/-$ animals. The cells differentiation in the BALF was analyzed by flow cytometry. Data are mean ± SEM (n = 6 mice per group). $^*P < 0.01$ $P2X_7R-/-$ OVA-DCs in wt versus WT-OVA-DCs in wt; $^{\#}P < 0.01$ $P2X_7R-/-$ OVA DCs in $P2X_7R-/-$ versus WT-OVA-DCs in $P2X_7R-/-$. One representative experiment out of three experiments is shown.

targeting $P2X_7R$ be a new option for the treatment of chronic airway inflammation. This hypothesis is further supported by our data showing that, compared with healthy control subjects, BALF macrophages from individuals with asthma produce higher levels of IL-1β, a cytokine involved in asthma pathogenesis (32–34), upon stimulation with the $P2X_7R$ agonist BzATP. We have recently shown that $P2X_7R$ expression is functionally up-regulated in neutrophils and macrophages of patients with COPD and that selective inhibition of $P2X_7R$ strongly suppresses smoke-induced lung inflammation and the development of emphysema (10 and Lucattelli and colleagues, unpublished).

Because $P2X_7R$ expression was up-regulated in lung tissue from mice with allergic airway inflammation, $P2X_7R$ signaling on structural cells might also be involved in the pathogenesis of allergic airway diseases. However, studies with bone marrow chimeras revealed that $P2X_7R$ deficiency on hematopoietic ($P2X_7R-/- \rightarrow$ wt) but not on structural (wt → $P2X_7R-/-$) cells was associated with reduced allergic lung inflammation. In line with these findings, the importance of $P2X_7R$ expression on hematopoietic cells has also been shown in smoke-induced lung inflammation (Lucattelli and colleagues, unpublished).

Increasing evidence points to an essential role of DCs in the pathogenesis of allergic airway inflammation (6). Several studies have demonstrated the functional expression of P2-receptor subtypes, including $P2X_7R$, on human and murine DCs (4, 12, 27, 35–38), and we recently reported that some of the proasthmatic effects of ATP *in vivo* are mediated via activation of purinergic receptors on myeloid DCs (2). Recruitment of myeloid DCs from the lung to the draining lymph nodes is an integral part of the primary and secondary immune responses to inhaled allergens (5, 39). Thus, we addressed whether functional $P2X_7R$ expression is necessary for the ATP-induced recruitment of blood DCs to the lungs and antigen-loaded DCs from the lung to the draining lymph nodes *in vivo*. The migratory capacity of DCs in response to ATP was not affected by $P2X_7R$ deficiency, in accordance with previous studies showing that the ATP-induced migration of various cells types is linked to the purinergic receptor subtype $P2Y_2$ (7, 8, 10, 40).

Priming of naive T cells into Th2 effector cells by myeloid DCs is a crucial step in asthma pathogenesis (5, 6), and ATP has been reported to enhance the Th2 priming capacity of DCs (4, 41). In line with a previous report by Mutini and colleagues (27), selective blocking of $P2X_7R$ or $P2X_7R$ deficiency of OVA-pulsed DCs was associated with a reduced capacity to prime OVA-specific naive T cells, suggesting an involvement of $P2X_7R$ signaling on DC in T-cell priming in allergic airway inflammation. To further support this hypothesis, we used a model in which allergic airway inflammation is induced by adoptive transfer of OVA-pulsed DCs to the lungs of naive animals followed by OVA challenge. Although wt animals receiving OVA-pulsed $P2X_7R-/-$ DCs showed attenuated allergic airway inflammation compared with animals receiving OVA-pulsed wt DCs, there was no difference in $P2X_7R-/-$ animals receiving OVA-pulsed wt DCs.

It is well established that stimulation of T cells by DCs via the CD80/CD86 complex is essential for the differentiation of

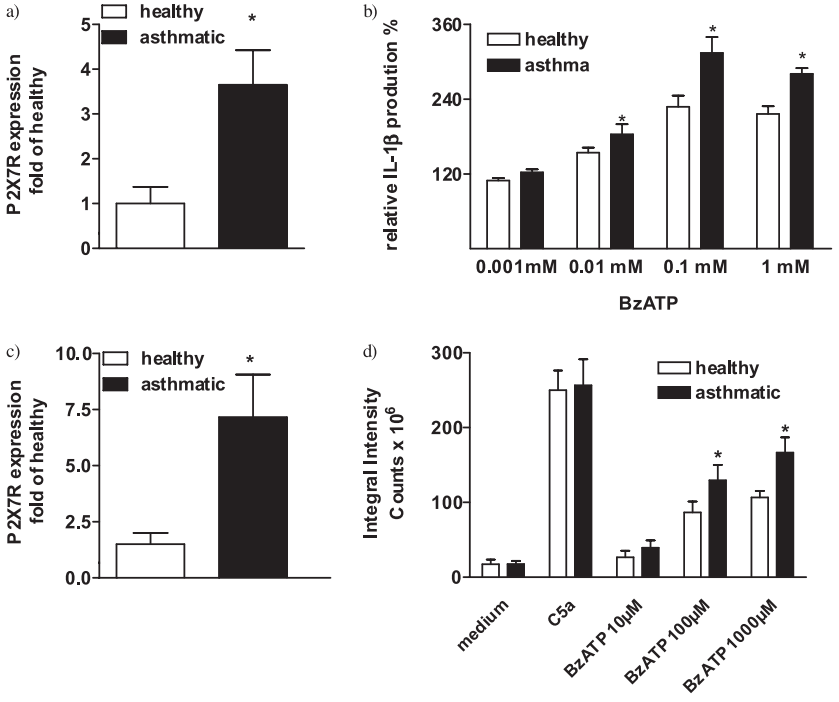

Figure 7. Functional up-regulation of P2X$_7$R expression on human BALF and blood eosinophils from individuals with asthma. (*a* and *b*) BALF macrophages were isolated from BALF from subjects with asthma (*black bars*; $n = 10$) and healthy control subjects (*white bars*; $n = 10$). (*a*) P2X$_7$R expression was analyzed by quantitative RT-PCR and is expressed as the amount of mRNA net to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Data are shown as mean ± SEM ($n = 10$). $P < 0.008$ (Mann-Whitney test). (*b*) Macrophages were cultured and stimulated with the P2X$_7$ agonist BzATP in different concentrations for 24 hours, and IL-1β levels measured in cell-free cell culture supernatants are shown as the release in % of baseline (medium control data are shown as mean ± SEM). *$P < 0.05$ for the comparison of subjects with asthma with healthy control subjects. (*c*) Eosinophils were isolated from blood from subjects with asthma (*black bars*; $n = 8$) and healthy control subjects (*white bars*; $n = 8$). (*a*) P2X$_7$R expression was analyzed by quantitative RT-PCR and is expressed as the amount of mRNA net to GAPDH. Data are shown as mean ± SEM ($n = 8$). $P < 0.004$ (Mann-Whitney test). (*d*) Oxygen radical production in response to BzATP or C5a. Data are shown as mean ± SEM (n = 8). *$P < 0.05$ for the comparison of subjects with asthma with healthy control subjects.

Th2 cells from naive T cells (3). ATP has been reported to increase the expression of the molecules CD80 and CD86 via P2R signaling (4, 41). Therefore, we speculated that an altered expression of these molecules by OVA-pulsed P2X$_7$R−/− DCs might be responsible for their decreased ability to prime naive T cells. However, as reported previously for fetal skin–derived DCs lacking P2X$_7$R, we observed no differences in the expression of costimulatory molecules in OVA-pulsed DCs derived from P2X$_7$R−/− deficient and wt mice (27).

Another mechanism contributing to DC-driven T-cell activation and proliferation is the production of mature IL-1β (15). Furthermore, it has been shown that, in addition to ATP, uric acid, another known inducer of IL-1β release by DCs, can promote Th2 polarizing by DCs (4, 23, 41, 42). In accordance with previous reports, we observed that DCs from P2X$_7$R−/− animals failed to release mature IL-1β upon stimulation with LPS or ATP (15). This is supported by previous research showing that IL-1β can restore the capacity of P2X$_7$R−/− DCs to stimulate antigen-specific T-cell proliferation (27).

The inhibition of P2X$_7$R can affect other hematopoietic cells involved in asthma pathogenesis. Indeed, P2X$_7$R signaling is involved in the activation and proliferation of T cells (18, 43) as well as in the release of proinflammatory mediators, such as reactive oxygen metabolites or IL-1β from eosinophils or macrophages. We observed a strong up-regulation of P2X$_7$R on human BAL macrophages and blood eosinophils from individuals with asthma associated with an increased capacity to produce IL-1β or reactive oxygen metabolites upon ATP stimulation.

In the extracellular space, ATP is metabolized rapidly by membrane-bound nucleotide-hydrolyzing enzymes, the so-called "ecto-nucleotidases" (NTPDase 1–8) (44). Adenosine has been found to be protective in an animal model of acute lung injury via the activation of A2b receptors (45). Furthermore, the generation of adenosine via ectonucleotidases by posthypoxic endothelial cells is critical for posthypoxia-associated protection of vascular permeability (46). However, proinflammatory cytokines (e.g., TNF-α) released at the site of inflammation suppress the activity of these ectonucleotidases (47). Therefore, although the ATP metabolite adenosine has potent antiinflammatory properties, there is good evidence that extracellular ATP in concentrations sufficient to activate P2X$_7$R is a potent proinflammatory signal.

In conclusion, the present study demonstrates that allergic airway inflammation in humans and mice is associated with the functional up-regulation of the P2X$_7$R on immune cells and that P2X$_7$R signaling (e.g., via modulating of DC function) is involved in the ATP-mediated proasthmatic effects. Although the majority of asthma patients can be treated with inhalative corticosteroids or β_2-agonists, there is still a subgroup of patients with asthma who need systemic steroids who are suffering from steroid-insensitive asthma. Thus, there is still a need for new treatment options (48). Because P2X$_7$R signaling is involved in the pathogenesis of acute and chronic asthma, targeting P2X$_7$R might be a new therapeutic option for the treatment of severe asthma. Because P2X$_7$R activation requires high doses of ATP and therefore occurs only in inflamed tissue (14, 15), P2X$_7$R antagonists should be well tolerated.

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