Purinergic Receptor Type 6 Contributes to Airway Inflammation and Remodeling in Experimental Allergic Airway Inflammation

Rodolfo Paula Vieira[1*], Tobias Müller[1*], Melanie Grimm[1], Volker von Gernler[1], Britta Vetter[1], Thorsten Dürk[1], Sanja Cicko[1], C. Korcan Ayata[1], Stephan Sorichter[1], Bernard Robaye[2], Robert Zeiser[3], Davide Ferrari[4], Andreas Kirschbaum[5], Gernot Zissel[1], J. Christian Virchow[6], Jean-Marie Boeynaems[2], and Marco Idzko[1]

[1]Department of Pneumology; [3]Department of Oncology and Hematology, and [5]Department of Thoracic Surgery, University Hospital Freiburg, Freiburg, Germany; [2]IRIBHM and Erasme Hospital, Université Libre de Bruxelles, Brussels, Belgium; [4]Section of General Pathology, Department of Experimental and Diagnostic Medicine, Interdisciplinary Center for the Study of Inflammation, University of Ferrara, Ferrara, Italy; and [6]Department of Pneumology, Clinic I/Center for Internal Medicine, University Hospital Rostock, Rostock, Germany

Rationale: **Extracellular nucleotides have recently been identified as proinflammatory mediators involved in asthma pathogenesis by signaling via purinergic receptors, but the role of the purinergic receptor type 6 (P2Y6R) has not been previously investigated.**

Objectives: **To investigate the role of P2Y6R in asthma pathogenesis.**

Methods: **Acute and chronic OVA model and also HDM model of allergic inflammation in C57Bl/6 mice treated with specific P2Y6R antagonist and P2Y6R$^{-/-}$ mice were evaluated for classical features of asthmatic inflammation. In addition, primary epithelial cell culture from human and epithelial cell lines from mouse and human were stimulated with P2Y6R agonist and treated with P2Y6R antagonist and assessed for IL-6, IL-8/CXCL8 and KC levels. Experiments with P2Y6R$^{-/-}$ and P2Y6R$^{+/+}$ chimera were performed to discriminate the role of P2Y6R activation in structural lung cells and in cells from hematopoietic system.**

Measurements and Main Results: **We observed that the intratracheal application of a P2Y6R antagonist (MRS2578) and P2Y6R deficiency inhibited cardinal features of asthma, such as bronchoalveolar lavage eosinophilia, airway remodeling, Th2 cytokine production, and bronchial hyperresponsiveness in the ovalbumin-alum model. MRS2578 was also effective in reducing airway inflammation in a model using house dust mite extracts to induce allergic lung inflammation. Experiments with bone marrow chimeras revealed the importance of the P2Y6R expression on lung structural cells in airway inflammation. In accordance with this finding, we found a strong up-regulation of P2Y6 expression on airway epithelial cells of animals with experimental asthma. Concerning the underlying mechanism, we observed that MRS2578 inhibited the release of IL-6 and IL-8/KC by lung epithelial cells *in vivo*, whereas intrapulmonary application of the P2Y6R agonist uridine-5′-diphosphate increased the bronchoalveolar levels of IL-6 and KC. In addition, selective activation of P2Y6 receptors induced the release of IL-6 and KC/IL-8 by murine and human lung epithelial cells *in vitro*.**

Conclusions: **P2Y6R expression on airway epithelial cells is up-regulated during acute and chronic allergic airway inflammation, and selective blocking of P2Y6R or P2Y6R deficiency on the structural cells reduces cardinal features of experimental asthma. Thus, blocking pulmonary P2Y6R might be a target for the treatment of allergic airway inflammation.**

Keywords: asthma; purinergic receptors; P2Y6 receptor; uridine diphosphate; cytokines

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Activation of purinergic receptors by nucleotides in cells of the hematopoietic system (e.g. eosinophils and dendritic cells) has emerged as a pivotal mechanism in asthma pathogenesis. However, the precise role of the purinergic receptor type 6 (P2Y6R) has been not yet been investigated.

What This Study Adds to the Field

This study provides evidence that P2Y6R is up-regulated during acute and chronic asthma and it is preferentially expressed on lung structural cells (airway epithelial cells). In addition, the present study demonstrates that P2Y6R is involved in acute and chronic allergic airway inflammation by regulating the secretion of IL-6 and IL-8 by airway epithelial cells.

Allergic asthma is one of the most common chronic diseases in western society, characterized by variable airway obstruction, bronchial hyperresponsiveness to nonspecific stimuli, mucus hypersecretion, and airway inflammation. Subepithelial deposition of extracellular matrix proteins, thickening of the airway wall, and goblet cell hyperplasia are typical features occurring in chronic asthma (1). The classic features of asthmatic airway inflammation include infiltration of the airway walls by eosinophils, Th2 cells, and mast cells. In addition to the mentioned cell types, dendritic cells (DCs) and lung structural cells (e.g., airway epithelial cells and/or fibroblasts) have also been demonstrated to play a role in asthma pathophysiology (1–3).

The inflammatory and structural changes of the asthmatic lung are induced by different mediators, including Th2 cytokines, prostanoids, and lipid mediators (3–5). Within the last few years, extracellular nucleotides, such as ATP, adenosine-5′-diphosphate, uridine-5′-triphosphate, or uridine-5′-diphosphate (UDP) have been established as potent immunomodulatory mediators (6, 7). Recently, we were able to demonstrate that nucleotides play a role in the initiation and maintenance of asthmatic airway inflammation by regulating the function of different hematopoietic cell types, such as DCs, eosinophils, and T cells (6–11). Nucleotides exert their cellular actions by binding to ionotropic P2X (P2X1–7) or metabotropic P2Y receptors (G-protein coupled receptors; P2Y1–14) (6–11).

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* These authors contributed equally to this work.

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Correspondence and requests for reprints should be addressed to Marco Idzko, M.D., Ph.D., University Hospital Freiburg, Department of Pneumology, Killianstrasse 5, 79106 Freiburg, Germany. E-mail: marco.idzko@uniklinik-freiburg.de

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The purinergic receptor subtype P2Y6 (P2Y6R) is a G-protein–coupled receptor that is selectively activated by the nucleotide UDP and selectively and pharmacologically blocked by MRS2578 (12–14). Transcripts for P2Y6R have been detected in several organs and cell types, including the intestines, spleen, thymus, blood leukocytes, aorta, lung, and DCs (12, 13). P2Y6R has been demonstrated to mediate proinflammatory effects, such as stimulation of cell proliferation, recruitment of neutrophils, and release of proinflammatory cytokines by intestinal epithelial cells (14–16), endolymphatic epithelia (17), or in airway epithelium (18). However, no study has yet investigated the precise role of P2Y6R in the context of asthmatic airway inflammation.

Here we show that P2Y6R expression is strongly up-regulated on airway epithelial cells of animals with acute and chronic allergic lung inflammation, contributing to asthma pathogenesis by increasing the release of proinflammatory cytokines by airway epithelial cells. Consequently, blocking of P2Y6R or P2Y6R deficiency resulted in a decreased acute and/or chronic allergic lung inflammation and airway remodeling.

METHODS

Animal Studies

Female C57BL/6 mice were bred at the animal facility of the University Hospital Freiburg under specific pathogen-free conditions. All experiments were approved by the local animal ethics committee.

Ovalbumin/alum model of acute and chronic allergic airway inflammation. Acute model: Female C57BL/6 mice, P2Y6R$^{-/-}$ and

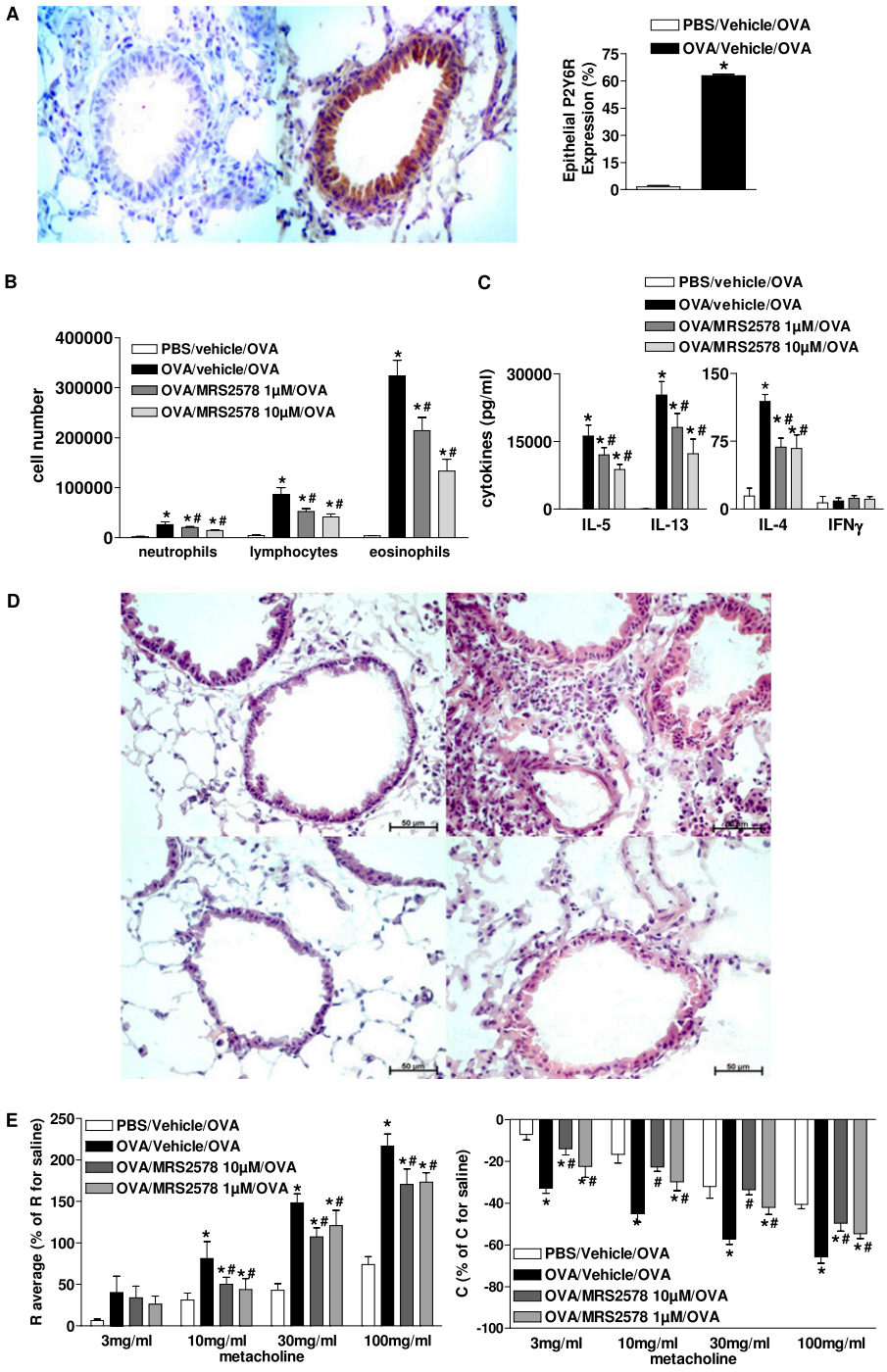

Figure 1. Selective inhibition of purinergic receptor type 6 (P2Y6R) suppresses acute allergic airway inflammation. To induce acute allergic airway inflammation, mice were sensitized by intraperitoneal injection of ovalbumin (OVA)/alum on Days 0 and 7 and were exposed on Days 19 to 21 to OVA aerosols. Before each aerosol, mice received an intratracheal injection of phosphate-buffered saline (PBS) or MRS2578. Groups are coded as sensitization/treatment/challenge. Twenty-four hours after last OVA aerosol challenge, animals were killed and the entire lung was collected. If not stated otherwise, one representative experiment out of three is shown (n = 5). Values are given as mean + SEM; $^{*}P < 0.05$ OVA/vehicle/OVA, OVA/MRS2578 1 μM/OVA and OVA/MRS2578 10 μM/OVA versus PBS/vehicle/OVA; $^{\#}P < 0.05$ OVA/MRS2578 1 μM/OVA and OVA/MRS2578 10 μM/OVA versus OVA/vehicle/OVA group. The expression of P2Y6R in sensitized and nonsensitized animals was detected by immunohistochemistry. (*A*) *Left photomicrograph*: sham-sensitized mice; *right photomicrograph*: OVA-sensitized mice. (*B*) Cell suspension in bronchoalveolar lavage fluid (BALF) was analyzed by flow cytometry. (*C*) Cytokines in cell culture supernatants of restimulated lymph nodes were measured by ELISA. (*D*) Hematoxylin and eosin staining of lung sections (*Upper left*: PBS/vehicle/OVA, *Upper right*: OVA/vehicle/OVA, *Lower left*: OVA/MRS2578 1 μM/OVA, *Lower right*: OVA/MRS2578 10 μM). (*E*) Bronchial hyperresponsiveness (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. Specifically to BHR, $^{*}P < 0.001$ compared with PBS/vehicle/OVA group and $^{\#}P < 0.01$ compared with OVA/vehicle/OVA group. One representative experiment out of three is shown.

$P2Y6R^{+/+}$ littermates on a C57Bl/6 background (n = 5 per group) were sham or ovalbumin (OVA) sensitized and challenged with OVA grade III (Sigma-Aldrich, Steinheim, Germany), as previously published (6). The generation of $P2Y6R^{-/-}$ animals was previously described (12); animals were backcrossed to C57bl/6 background for at least eight generations. Briefly, mice were OVA or sham sensitized by intraperitoneal injection of OVA/alum or phosphate-buffered saline (PBS)/alum on Days 0 and 7 and were challenged with OVA aerosols on Days 17 to 19 (6). Thirty minutes before each allergen challenge, animals were anesthetized with ketamine and xylazine and given an intratracheal injection of control vehicle or the receptor antagonist MRS2578 (Tocris, Ellisville, MO) or the agonist UDP (Sigma-Aldrich, Steinheim, Germany). Experiments were repeated three times.

Chronic model: Female C57BL/6 mice (6–9 wk, n = 8 per group) were sham or OVA sensitized by intraperitoneal injection on Day 0 and 7 and subsequently challenged with OVA aerosols three times weekly for 8 weeks (8). Treatment with MRS2578 was performed three times weekly for the last 2 weeks of OVA aerosol challenge, 30 minutes before each allergen challenge. Experiments were repeated three times.

Twenty-four hours after the last OVA exposure, in both acute and chronic OVA models, measurement of airway hyperresponsiveness, fluorescence-activated cell sorter analysis of the bronchoalveolar lavage fluid (BALF), and lung resection for histology and immunohistochemistry were performed as previously described (6, 8, 9, 19, 20). The levels of cytokines were measured in BALF and in restimulated mediastinal

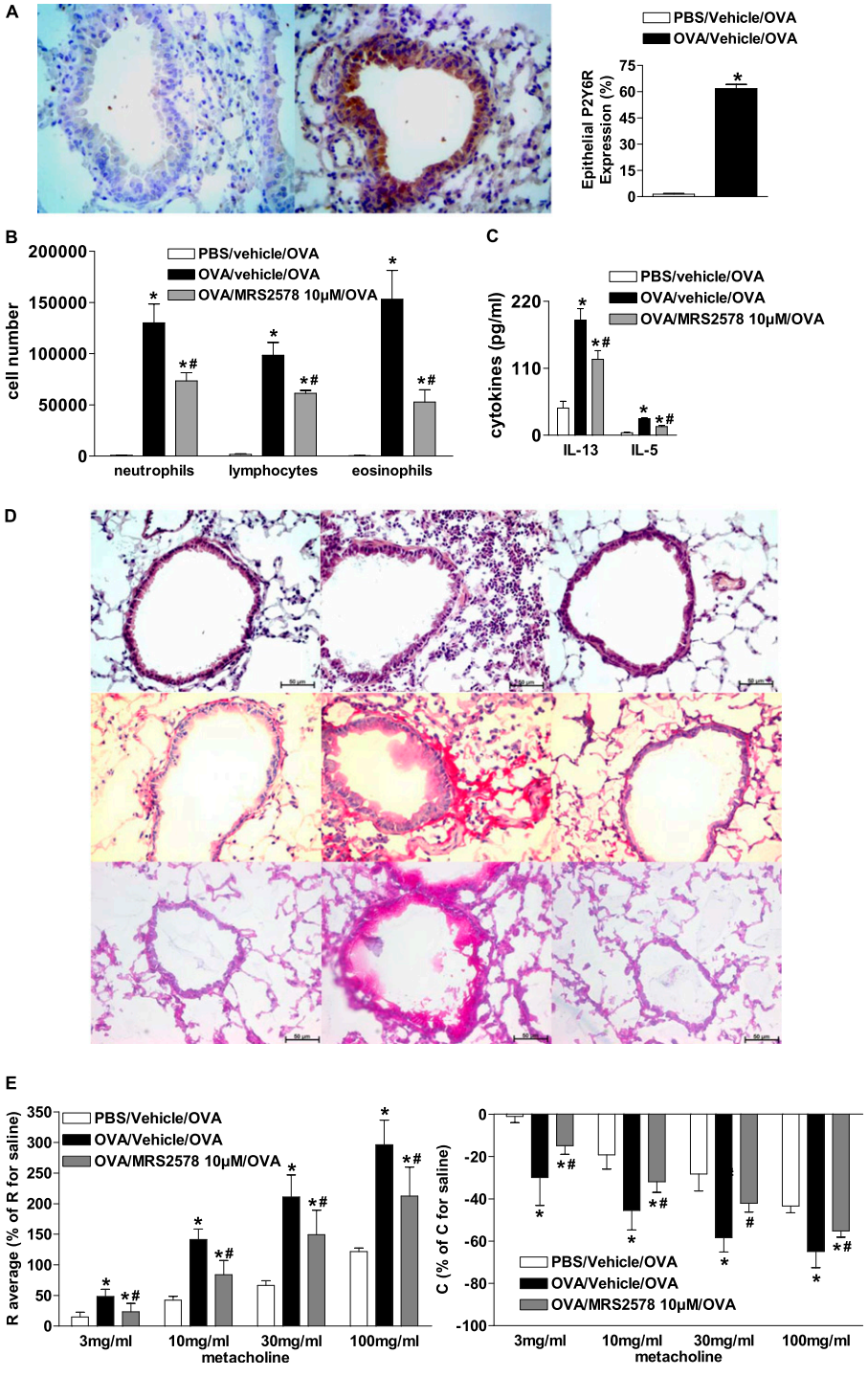

Figure 2. Purinergic receptor type 6 (P2Y6R) inhibition reduces progressive airway inflammation and airway remodeling in a model of chronic experimental asthma. For the induction of chronic experimental allergic airway inflammation, animals were sensitized to ovalbumin (OVA) by intraperitoneal injection on Day 0 and 7 and afterward challenged with OVA aerosols three times per week for 8 weeks. Treatment with MRS2578 was performed during the last 2 weeks of OVA aerosol challenge. Groups are coded as sensitization/treatment/challenge. One representative experiment out of three is shown (n = 8). Values are given as mean + SEM; $^{*}P < 0.05$, OVA/vehicle/OVA and OVA/MRS2578 10 μM/OVA versus phosphate-buffered saline (PBS)/vehicle/OVA; $^{\#}P < 0.05$, OVA/MRS2578 10 μM/OVA versus OVA/vehicle/OVA group. The expression of P2Y6R in sensitized and nonsensitized animals was detected by immunohistochemistry. (*A*) *Left photomicrograph*: sham-sensitized mice; *right photomicrograph*: OVA-sensitized mice. (*B*) Bronchoalveolar lavage fluid (BALF) cell differential counts analyzed by flow cytometry. (*C*) BALF cytokines measured by ELISA. (*D*) Hematoxylin and eosin staining for inflammation (*upper panel*), Sirius red-staining for collagen-deposition (*middle panel*), and periodic acid Schiff staining for goblet cell hyperplasia/mucus production (*lower panel*); *Left*: PBS/vehicle/OVA, *Middle*: OVA/vehicle/OVA, *Right*: OVA/MRS2578 10 μM/OVA. (*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. One representative experiment out of three is shown.

lymph nodes (MLN). For details about the airway hyperresponsiveness method, cytokine measurement, and histology and immunohistochemistry, *see* online supplement.

House dust mite–induced allergic airway inflammation. Female C57BL/6 mice (6–9 wk, n = 5 per group) were injected intratracheally with 100 μg *Dermatophagoides pteronyssinus* extract (Greer Laboratories, Lenoir, NC) dissolved in 80 μl PBS on Day 0, Day 7, and Day 14. In the MRS2578-treated group, house dust mite extract was admixed with MRS2578 on Days 7 and 14. Animals were assessed for the classic features of asthma, such as airway hyperresponsiveness, inflammation, and remodeling, and cytokine levels in restimulated cells of MLN on day 17, as previously described (6, 8, 9). Experiments were repeated three times. For details, *see* online supplement.

Animal studies using P2Y6R$^{-/-}$ and P2Y6R wild-type chimera. Generation of chimeric recipients with P2Y6R deficiency of hematopoietic cells: After lethal irradiation with 900 cGy (2 × 450 cGy), wild-type (C57Bl/6, WT) and P2Y6$^{-/-}$ recipients were intravenously given 5 × 10^6 P2Y6R$^{-/-}$ or WT bone marrow cells. The following donor/recipient pairs were combined: WT → WT (OVA sensitized), WT → P2Y6R$^{-/-}$ (OVA sensitized), P2Y6R$^{-/-}$ → WT (OVA sensitized), and P2Y6R$^{-/-}$ → P2Y6R$^{-/-}$ (OVA sensitized). In all chimera experiments six to eight animals were used per group. Animals were assessed for the classic features of asthma, such as airway inflammation and cytokine levels in restimulated cells of MLN.

In Vitro Studies

Isolation of primary normal human bronchial epithelial cells. The normal human bronchial epithelial cells were obtained from the bronchi of explanted lungs or their bronchial rings. The study was approved by the local ethics committee of Freiburg. Bronchi were longitudinally opened and mechanically dissected by using a disposable scalpel and subsequently washed in ice-cold Hanks' balanced salt solution. The mucosa was minced in small pieces and digested in Dispase II in 80 ml PII solution, supplemented with 100 μl DNase and penicillin and streptomycin for 90 minutes in a water bath at 37°C. The crude solution was filtered using a cell strainer of 100 μm, centrifuged at 1,500 rpm, 5 minutes at 4°C and then resuspended in RPMI 1649 medium supplemented with penicillin and streptomycin for 15 minutes and placed in a Petri dish for 15 minutes. Nonadherent cells were carefully harvested and counted and then cultured (1 × 10^6 cells/well) in 6-well plates using Quantum 286 for Epithelial Cells medium (PPA Laboratories GmbH, Cölbe, Germany). The cells were rested for 24 hours. Then the medium was changed and the cells were stimulated with the indicated concentrations of UDP and MRS2578. After 24 hours, cell culture supernatants were collected for cytokine measurements by ELISA.

Human and mouse epithelial cell lines A549, BEAS-2B, and LA-4. Human cell line cells (BEAS-2B) were cultured in RPMI 1640 (Gibco, Carlsbad, CA), supplemented with 10% fetal calf serum (FCS), 100 U/ml gentamicin, and 1% glutamine. LA-4 murine

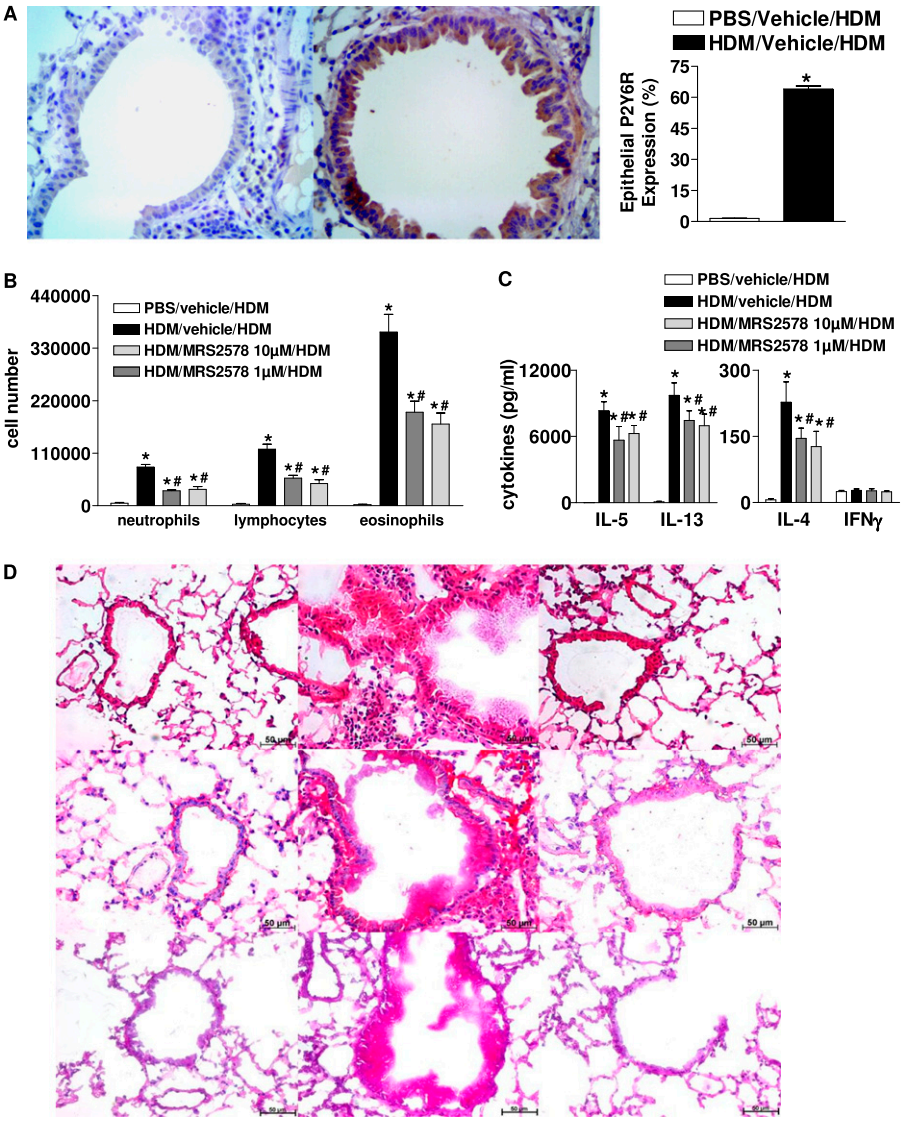

Figure 3. MRS2578 reduces house dust mite–induced allergic airway inflammation. For the house dust mite extract (HDM)-induced asthma model, mice were injected intratracheally with 100 μg *Dermatophagoides pteronyssinus* extract on Day 0, Day 7, and Day 14 and were assessed for the classical features of allergic airway inflammation on Day 17. Groups are coded as sensitization/treatment/challenge. One representative experiment out of three is shown (n = 5). Values are given as mean + SEM; *$P < 0.05$, HDM/vehicle/HDM, HDM/MRS2578 10 μM/HDM and HDM/MRS2578 1 μM/HDM versus phosphate-buffered saline (PBS)/vehicle/HDM: #$P < 0.05$, HDM/MRS2578 10 μM/HDM and HDM/MRS2578 1 μM/HDM versus HDM/vehicle/HDM. The expression of purinergic receptor type 6 (P2Y6R) in sensitized and nonsensitized animals was detected by immunohistochemistry. (*A*) *Left photomicrograph*: sham-sensitized mice; *right photomicrograph*: ovalbumin (OVA)-sensitized mice. (*B*) Bronchoalveolar lavage fluid cell differential counts analyzed by flow cytometry. (*C*) Cytokines in cell culture supernatants of restimulated lymph nodes measured by ELISA. (*D*) Hematoxylin and eosin staining for inflammation (*upper panel*), Sirius red staining for collagen deposition (*middle panel*), and periodic acid Schiff staining for goblet cell hyperplasia/mucus production (*lower panel*); *Left*: PBS/vehicle/HDM, *Middle*: HDM/vehicle/HDM, *Right*: HDM/MRS2578 10 μM/HDM. One representative experiment out of three is shown.

bronchial epithelial cells (American Type Culture Collection, Rockville, MD) were cultured in F12K Nutrient Mixture (Gibco) supplemented with 15% FCS, 100 U/ml gentamicin, and 1% glutamine. A549 cells (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) were grown in Eagle's minimum essential medium (Gibco, Paisley, UK) supplemented with 5% FCS, 100 U/ml gentamicin, and 1% glutamine. For each experiment, 1×10^6 cells were seeded into 24-well plates and rested for 24 hours. Then medium was changed and the cells were stimulated with the indicated concentrations of UDP and MRS2578. After 24 hours, cell culture supernatants were collected for cytokine measurements by ELISA.

Cytokine measurements. The levels of IL-4, IL-5, IL-6, IL-8/CXCL8, IL-13, KC, and IFN-γ were measured using commercially available ELISA kits (R&D Systems, Minneapolis, MN) and performed according to the manufacturer's recommendations.

Statistical Analysis

If not stated otherwise, groups were compared using analysis of variance, followed by Bonferroni comparison test. Probability values of $P < 0.05$ were regarded as significant.

RESULTS

Acute Allergic Airway Inflammation Increases P2Y6R Expression on Airway Epithelial Cells

For the induction of acute allergic airway inflammation, mice were sensitized to OVA with alum and challenged 10 days later with three sequential daily aerosolizations of OVA, which led to the characteristic features of allergic airway inflammation (6). One day after the last challenge, animals were killed and the lungs were stained for the expression of P2Y6R. As shown in Figure 1A, 24 hours after the last challenge P2Y6R expression in the airway epithelium was strongly increased in OVA but not in PBS-challenged mice.

Selective Inhibition of P2Y6R Suppresses Acute Allergic Airway Inflammation

To better understand the functional relevance of the strong up-regulation of P2Y6R expression, the selective P2Y6R antagonist MRS2578 was administered intratracheally to OVA-sensitized mice 30 minutes before each allergen challenge. MRS2578 dose-dependently reduced the number of BALF eosinophils, lymphocytes, and neutrophils (Figure 1B), as well as the production of IL-4, IL-5, and IL-13 by mediastinal lymph nodes (Figure 1C), whereas IL-10 (data not shown) and IFN-γ production were not changed. This was accompanied by a decrease in peribronchiolar and perivascular inflammation (Figure 1D). Additionally, MRS2578-treated animals showed reduced bronchial hyperresponsiveness toward methacholine compared with vehicle-treated animals as determined 24 hours after the last allergen challenge by invasive measurement of dynamic resistance and compliance in mechanically ventilated mice (Figure 1E). Additionally, MRS2578 had no effect on resistance and compliance of sham-sensitized OVA-challenged animals.

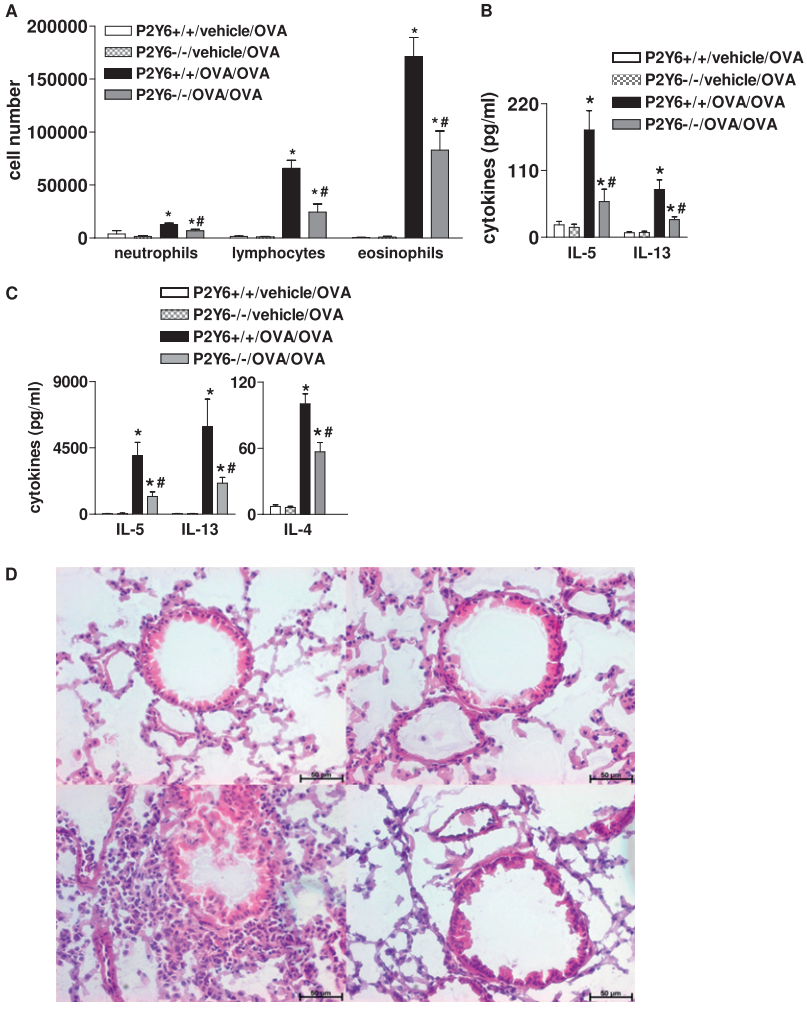

Figure 4. Purinergic receptor type 6 (P2Y6R) deficiency suppresses the development of allergic airway inflammation. To induce acute allergic airway inflammation, the same protocol of ovalbumin (OVA)-induced acute lung inflammation described above was followed. If not stated otherwise, one representative experiment out of three is shown (n = 5). Values are given as mean + SEM; $*P <$ 0.05, $P2Y6^{+/+}$ and $P2Y6^{-/-}$/vehicle/OVA versus $P2Y6^{+/+}$ and $P2Y6^{-/-}$/OVA/OVA; $^{\#}P < 0.05$, $P2Y6^{+/+}$/OVA/OVA versus $P2Y6^{-/-}$/OVA/OVA. (*A*) Cell suspension in bronchoalveolar lavage fluid (BALF) was analyzed by flow cytometry. (*B*) Cytokines in BALF were measured by ELISA. (*C*) Cytokines in cell culture supernatants of restimulated lymph nodes were measured by ELISA. (*D*) Hematoxylin and eosin staining of lung sections (*Upper left*: P2Y6WT/vehicle/OVA, *Upper right*: P2Y6KO/vehicle/OVA, *Lower left*: P2Y6WT/OVA/OVA, *Lower right*: P2Y6KO/OVA/OVA). One representative experiment out of three is shown.

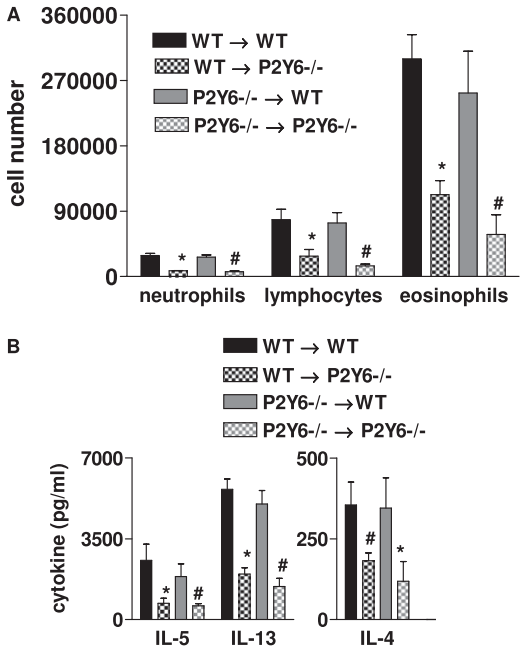

Figure 5. Purinergic receptor type 6 (P2Y6R)$^{-/-}$ and wild-type (WT) chimera reveal the importance of P2Y6R on structural cells in the reduction of allergic airway inflammation. After irradiation with 2 × 450 cGy, WT recipients (C57Bl/6, WT) and P2Y6$^{-/-}$ were given 5 × 10^6 bone marrow cells of P2Y6R$^{-/-}$ or WT donors intravenously. The following bone marrow chimeras were generated: WT → WT (ovalbumin [OVA] sensitized), WT → P2Y6R$^{-/-}$ (OVA sensitized), P2Y6R$^{-/-}$ → WT (OVA sensitized), and P2Y6$^{-/-}$ → P2Y6$^{-/-}$ (OVA sensitized). In all chimera experiments six to eight animals per group were used. Values are given as mean + SEM; *$P < 0.05$, WT → P2Y6$^{-/-}$ versus WT → WT; #$P < 0.05$, P2Y6$^{-/-}$ → P2Y6$^{-/-}$ versus P2Y6$^{-/-}$ → WT. (*A*) Cell suspension in bronchoalveolar lavage fluid was analyzed by flow cytometry. (*B*) Cytokines in cell culture supernatants of restimulated lymph nodes were measured by ELISA. One representative experiment out of three is shown.

P2Y6R Inhibition Reduces Progressive Airway Inflammation and Airway Remodeling in a Model of Chronic Experimental Asthma

Next we questioned whether P2Y6R signaling is also involved in chronic allergic airway inflammation. Therefore, we first analyzed the expression of P2Y6R in the lungs of animals chronically exposed to OVA (three times per wk, 8 wk). As shown in Figure 2A, in this model of chronic allergic airway inflammation P2Y6R expression on airway epithelium was strongly up-regulated. To test the functional relevance of this up-regulation, OVA-sensitized mice were challenged on 3 days per week over a time period of 8 weeks. Treatment with the selective P2Y6R antagonist MRS2578 was performed during the last 2 weeks of the protocol before allergen challenge. As shown in Figure 2B, vehicle-treated OVA-sensitized mice showed an increase in BALF eosinophils, neutrophils, and lymphocytes as compared with sham-sensitized animals. This was accompanied by higher levels of the cytokines IL-5 and IL-13 in BALF (Figure 2C), increased airway remodeling (Figure 2D), and an increased responsiveness to inhaled methacholine (Figure 2E). Intrapulmonary treatment with MRS2578 (10 μM) reduced BALF eosinophilia and the levels of IL-5 and IL-13 in the BALF and led to a markedly attenuated change in methacholine responsiveness after OVA challenge. Evaluation of the lung histology revealed a marked reduction of peribronchial and perivascular inflammation as well as goblet cell hyperplasia, mucus production, and subepithelial fibrosis as evidenced by decreased deposition of collagen fibers on the airways wall (Figure 2E). Additionally, MRS2578 had no effect on resistance and compliance of sham-sensitized OVA-challenged animals.

MRS2578 Inhibits House Dust Mite–Induced Allergic Airway Inflammation

The OVA-alum model has been used extensively to study asthmatic airway inflammation *in vivo*. However, to get a closer resemblance to human asthma, we additionally used another animal model in which eosinophilic airway inflammation is induced by house dust mite extract. Intratracheal administration of house dust mite extract on Day 0, Day 7, and Day 14 resulted in a strong up-regulation of P2Y6R in the airway epithelium (Figure 3A) and an increase in BAL eosinophils, lymphocytes, and neutrophils (Figure 3B), Th2 cytokine production by MLN cells (Figure 3C), tissue infiltration by leukocytes, collagen deposition in the airway wall, and mucus hyperproduction (Figure 3D). However, when administered together with the allergen MRS2578 decreased airway inflammation, as determined by a reduction in total cells and eosinophils in BALF. In addition, Th2 cytokine production by MLN and peribronchial and perivascular inflammation were reduced, as was collagen deposition and mucus production.

P2Y6R Deficiency Results in Decreased Airway Inflammation and Remodeling

As a proof of concept, we investigated whether P2Y6R deficiency is associated with reduced allergic airway inflammation. We

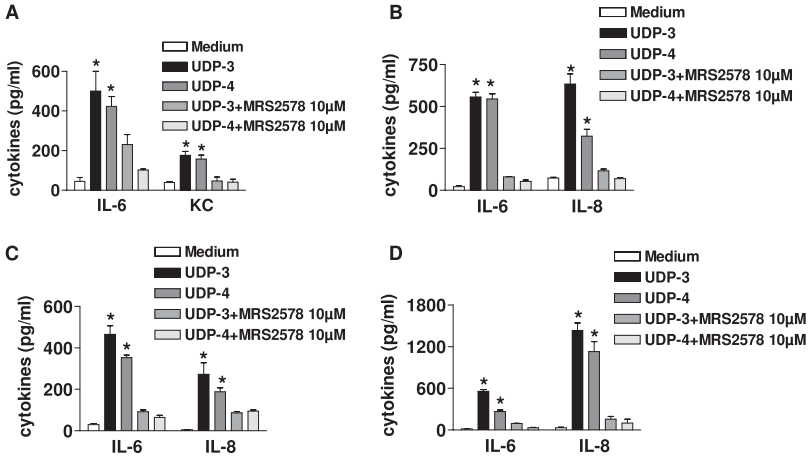

Figure 6. Uridine-5′-diphosphate (UDP) induces IL-6 and KC/IL-8 release by airway epithelial cells *in vitro*. (*A*) Levels of IL-6 and KC in supernatant of LA-4 (1 × 10^6 cells/well) stimulated for 24 hours with indicated concentrations of UDP and MRS2578. (*B*) Levels of IL-6 and IL-8 in supernatant of A549 (1 × 10^6 cells/well) stimulated for 24 hours with indicated concentrations of UDP and MRS2578. (*C*) Levels of IL-6 and IL-8 in supernatant of BEAS-2B (1 × 10^6 cells/well) stimulated for 24 hours with indicated concentrations of UDP and MRS2578. (*D*) Levels of IL-6 and IL-8 in supernatant of primary normal bronchial epithelial cells (1 × 10^6 cells/well) stimulated for 24 hours with indicated concentrations of UDP and MRS2578. One representative experiment out of three is shown. Values are given as mean + SEM; *$P < 0.05$, UDP-3 and UDP-4 vs. medium, UDP-3 + MRS2578 10 μM and UDP-4 + MRS2578 10 μM.

observed that P2Y6R deficiency results in a decreased number of eosinophils, lymphocytes, and neutrophils in BALF (Figure 4A) and also in a decreased peribronchial and periarterial inflammation (Figure 4D). P2Y6R deficiency also resulted in decreased levels of IL-5 and IL-13 in BALF (Figure 4B) as well as the IL-4, IL-5, and IL-13 production in restimulated MLN cells (Figure 4C).

P2Y6R$^{-/-}$ and WT Chimera and the Relevance of P2Y6R on Structural Cells in the Reduction of Allergic Airway Inflammation

To discriminate the role of P2Y6R signaling on hematopoietic and nonhematopoietic/structural cells in allergic inflammation, hematopoietic cells of WT and P2Y6$^{-/-}$ animals were depleted by irradiation and subsequently reconstituted with bone marrow of P2Y6$^{-/-}$ and WT mice. These chimeric mice were sensitized with OVA alum as described above. As shown in Figure 5, WT animals reconstituted with bone marrow of WT or P2Y6R$^{-/-}$ were not protected against the development of allergic airway inflammation induced by OVA, as observed in unchanged BAL eosinophilia (Figure 5A) and Th2 cytokines in MLN (Figure 5B). On the other hand, when P2Y6$^{-/-}$ animals were reconstituted with bone marrow of WT or P2Y6R$^{-/-}$ they still presented a protection, as evidenced by decreased BAL eosinophilia (Figure 5A) and Th2 cytokines in MLN (Figure 5B).

UDP Induces IL-6, KC/IL-8 Release by Lung Epithelial Cells *In Vitro*

Production of cytokines and chemokines (such as IL-6 and IL-8/CXCL8) by lung structural cells (e.g., airway epithelial cells) has been shown to influence the outcome of the allergic airway inflammation. In this regard, we investigated whether the activation of P2Y6R by UDP could regulate the production of IL-6 and IL-8/CXCL8 by the mouse bronchial epithelial cell line LA-4, by the lung alveolar lung epithelial cell line A549, by the human lung airway epithelial cell line BEAS-2B, and also by primary normal human bronchial epithelial cells. As shown in Figures 6A–6D, UDP dose-dependently induced the release of IL-6, KC, and IL-8, respectively, by LA-4, A549, BEAS-2B, and primary normal human bronchial epithelial cells. This production was completely blocked by pretreatment of the cells with the selective P2Y6R antagonist MRS2578 (Figures 6A–6D, respectively).

P2Y6R Signaling Regulates KC and IL-6 Production *In Vivo*

To verify whether P2Y6R-mediated KC and IL-6 production from airway epithelial cells is involved in the induction of acute allergic airway inflammation, we measured the levels of KC and IL-6 in BALF of OVA-sensitized mice treated with MRS2578 or vehicle before allergen challenge. As shown in Figure 7A, intrapulmonary antagonization of P2Y6R was associated with a strong reduction of IL-6 and KC levels in BALF. To prove that this reduction is indeed a consequence of P2Y6R signaling and not due to a general reduction in inflammation we administrated exogenous UDP or vehicle to the lungs of animals with acute and chronic allergic airway inflammation or to sham-sensitized animals; 24 hours later BALF levels of IL-6 and KC were analyzed: in the BALF of UDP-treated animals, higher levels of IL-6 and KC were detected both in acute and chronic OVA-alum models compared with vehicle-treated animals (Figures 7B and 7C, respectively).

DISCUSSION

In the present study, we provide evidence that P2Y6R signaling on airway epithelial cells is involved in the pathogenesis of allergic airway inflammation in mouse models of allergic asthma wherein P2Y6R-mediated signaling contributed to acute and chronic allergic airway inflammation.

Recently it has been reported that P2Y2R and P2X7R signaling on hematopoietic cells (such as eosinophils and DCs) contributes to the development of allergic airway inflammation (8, 9). Functional expression of P2Y6R has also been described on DCs (13), eosinophils (7), mast cells (21), monocytes (22, 23), and neutrophils (24), suggesting that P2Y6R might also be involved in the pathogenesis of allergic airway inflammation by affecting the function of these hematopoietic cells. We therefore investigated the effect of an intratracheal application of the P2Y6R-specific antagonist MRS2578 in different mouse models of asthma. Indeed, MRS2578 reduced the several features of asthma in the acute and chronic OVA-alum model as well as in a model wherein allergic airway inflammation had been induced by a house dust mite extract. Similarly, P2Y6R deficiency confirmed the importance of P2Y6R in the modulation of allergic inflammation, because P2Y6R-deficient animals sensitized and challenged with OVA presented decreased numbers of eosinophils, lymphocytes, neutrophils, and macrophages in BALF, as well as a decreased production of IL-4, IL-5, and IL-13 by restimulated cells of mediastinal lymph nodes.

However, our experiments with bone marrow chimera showed that P2Y6R deficiency of hematopoietic cells (P2Y6R$^{-/-}$ → WT) is not associated with reduced allergic airway inflammation, whereas reconstitution of P2Y6R$^{-/-}$ animals with WT bone marrow cells are associated with reduced allergic airway inflammation. Thus, the observed effects of P2Y6R signaling seem to

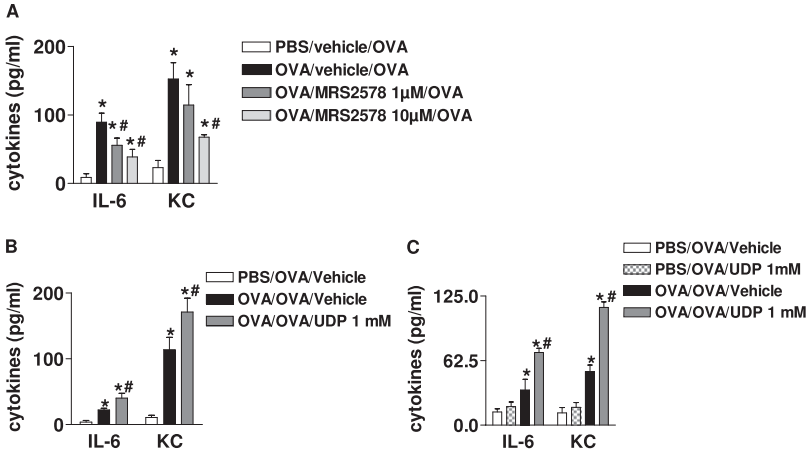

Figure 7. Purinergic receptor type 6 (P2Y6R) signaling regulates KC and IL-6 production *in vivo*. (*A*) Levels of IL-6 and KC measured in the bronchoalveolar lavage fluid (BALF) of acute ovalbumin (OVA)-sensitized mice treated with MRS2578 10 μM or vehicle before allergen challenge. (*B*) IL-6 and KC levels in BALF of acute OVA-sensitized mice treated with uridine-5′-diphosphate (UDP) or vehicle after allergen challenge. (*C*) Levels of IL-6 and KC in BALF of chronic OVA-sensitized mice treated with UDP or vehicle after allergen challenge. One representative experiment out of three is shown. Values are given as mean + SEM; $^{*}P < 0.05$, OVA/vehicle/OVA, OVA/MRS2578 1 μM/OVA and OVA/MRS2578 10 μM/OVA vs. phosphate-buffered saline (PBS)/vehicle/OVA and PBS/OVA/vehicle; and $^{\#}P < 0.05$, OVA/MRS2578 1 μM/OVA, OVA/MRS2578 10 μM/OVA and OVA/OVA/UDP 1 mM vs. OVA/vehicle/OVA.

be mediated by structural lung cells rather than hematopoietic cells. In line with this finding, immunohistochemical staining of lung sections revealed that P2Y6R is mainly expressed on airway epithelial cells. Indeed, we also observed a weak P2Y6R staining (compared with intensity of P2Y6R staining on airway epithelial cells) on other structural cells (i.e., smooth muscle and endothelial cells), which is in agreement with recent studies demonstrating the presence of P2Y6R on smooth muscle and on endothelial cells (28, 29). Thus structural cells might also contribute P2Y6R-mediated effects to allergic airway inflammation.

Airway epithelial cells have previously been shown to play an important role in asthma pathophysiology via the interaction with inflammatory cells and the secretion of proinflammatory cytokines (25–27). Some epithelial-derived cytokines, such as IL-8 and IL-6, have been associated not only with inflammation but also with airway remodeling and hyperresponsiveness (1, 4, 25–27). Thus we addressed whether P2Y6R signaling can regulate the release of IL-6 or IL-8/KC from mouse or human airway epithelial cells *in vitro*. Indeed, P2Y6R agonist UDP stimulated the production of IL-6 and IL-8/KC by airway epithelial cells in both human and murine cell lines. This effect was completely abrogated when cells were pretreated with the selective P2Y6R antagonist MRS2578 before stimulation. These results are in line with a previous report by Bar and colleagues, showing that P2Y6R signaling induces IL-6 release in macrophages (15). In addition, ATP has been shown to induce the release of IL-8/CXCL8 from human airway epithelial cells (HBE1) (28); however, the exact purinergic receptor subtypes mediating this response were unknown (28). Besides, Grbic and colleagues observed that intestinal inflammation results in an up-regulation of P2Y6R expression on gut epithelial cells, which was associated with an increased capacity to release IL-8 on stimulation with the P2Y6R agonist UDP (19). Measuring these cytokines in a mouse model of acute airway inflammation, we detected a significant increase in the BALF levels of IL-6 and KC. This increase was further enhanced by the intrapulmonary application of UDP, whereas MRS2578 treatment significantly decreased BALF levels of IL-6 and KC. Taken together, these data suggest that P2Y6R signaling contributes to acute allergic airway inflammation via modulating epithelial cell–derived IL-6 and IL-8/KC production.

In addition to the acute allergic airway inflammation, MRS2578 was also able to attenuate the morphological changes occurring in chronic asthma. The degree of structural changes in asthmatic airways is of high significance as it is directly correlated with disease severity and remains a major unresolved problem in asthma control (29). In the present study we demonstrated that the selective blockade of P2Y6R results in a decreased collagen deposition and reduced thickness of airway epithelium and smooth muscle layer, which was paralleled by a decreased bronchial hyperresponsiveness. As in the acute allergic airway inflammation, we could also detect increased production of IL-6 and KC in animals with chronic asthma after intratracheal application of UDP. Beyond acute inflammation, these cytokines are also associated with chronic inflammatory and fibroproliferative disorders, including chronic asthma (29, 30). KC has been found to be important for the proliferation of smooth muscle cells; IL-6 has similar effects (29, 30). Therefore, it seems likely that the structural changes mediated by P2Y6R are also triggered by the release of these cytokines.

In summary, we demonstrated for the first time that the purinergic receptor subtype P2Y6 is involved in pathogenesis of both acute and chronic allergic airway inflammation in a mouse model of asthma by modulating the production of IL-6 and IL-8 by airway epithelial cells. Therefore, further studies in humans are needed to investigate if selective P2Y6R antagonists can be a new promising approach for the treatment of allergic airway inflammation and airway remodeling.

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