

Cyclooxygenase-2/Prostaglandin D₂/CRTH2 Pathway Mediates Double-Stranded RNA-Induced Enhancement of Allergic Airway Inflammation¹

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Respiratory RNA viruses responsible for the common cold often worsen airway inflammation and bronchial responsiveness, two characteristic features of human asthma. We studied the effects of dsRNA, a nucleotide synthesized during viral replication, on airway inflammation and bronchial hyperresponsiveness in murine models of asthma. Intratracheal instillation of poly I:C, a synthetic dsRNA, increased the airway eosinophilia and enhanced bronchial hyperresponsiveness to methacholine in OVA-sensitized, exposed rats. These changes were associated with induction of cyclooxygenase-2 (COX-2) expression and COX-2-dependent PGD₂ synthesis in the lungs, particularly in alveolar macrophages. The direct intratracheal instillation of PGD₂ enhanced the eosinophilic inflammation in OVA-exposed animals, whereas pretreatment with a dual antagonist against the PGD₂ receptor (CRTH2) and the thromboxane A₂ receptor, but not with a thromboxane A₂ receptor-specific antagonist, nearly completely eliminated the dsRNA-induced worsening of airway inflammation and bronchial hyperresponsiveness. CRTH2-deficient mice had the same degree of allergen-induced airway eosinophilia as wild-type mice, but they did not exhibit a dsRNA-induced increase in eosinophil accumulation. Our data demonstrate that COX-2-dependent production of PGD₂ followed by eosinophil recruitment into the airways via a CRTH2 receptor are the major pathogenetic factors responsible for the dsRNA-induced enhancement of airway inflammation and responsiveness. *The Journal of Immunology*, 2008, 180: 541–549.

Respiratory infections with RNA viruses, such as rhinovirus, coronavirus, parainfluenza, or respiratory syncytial virus, are associated with asthmatic exacerbations (1), however, the mechanism of these changes has not been established. In addition to neutrophilic and lymphocytic airway inflammation observed in both healthy and asthmatic subjects during viral infection (2, 3), several lines of evidence suggest that eosinophils also play an important role in patients with asthma during viral infection-induced exacerbation (4–8). However, little is known regarding the mechanisms of recruitment and activation of eosinophils in asthmatic airways during viral infection.

Lipid mediators, such as PGs, leukotrienes, and platelet-activating factor, have been considered essential in the development of

tissue eosinophilia during allergic inflammation (9–12). Recent studies have shown that PGD₂, a major cyclooxygenase (COX)³ metabolite synthesized in activated mast cells and macrophages, is released during early and late asthmatic responses following allergen exposure (13, 14), and acts as a potent chemoattractant of eosinophils in vitro (15, 16) and in vivo (17, 18). We have reported that COX-2 inhibitors, which stop the synthesis of PGD₂ in the lungs of allergen-sensitized and exposed guinea pigs, attenuated the accumulation of eosinophil in the airways (19). Others have reported that the overexpression of PGD synthase or inhalation of aerosolized PGD₂ enhanced the allergen-induced airway eosinophilia in mice (20, 21).

The bioactivity of PGD₂ is mediated by two G protein-coupled receptors, DP1 and CRTH2 (DP2), both of which are expressed on the surface of eosinophils (22). The genetic disruption of the DP1 receptor, or its pharmacological blockade, lowered the number of eosinophils in allergen-induced inflammation of the airways in mice (23) and in guinea pigs (24). However, a DP1-specific agonist, BW 245C, failed to replicate the effects of PGD₂ on the mobilization of eosinophils in vitro (15, 16) and had an attenuating effect on the inflammation of the airways in vivo (25). CRTH2, another PGD₂ receptor identified as a molecule preferentially expressed on Th2 lymphocytes, eosinophils, and basophils, transduces the chemokinetic

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³ Abbreviations used in this paper: COX, cyclooxygenase; SPF, specific pathogen free; VAF, virus Ab free; BN, Brown Norway; BAL, bronchoalveolar lavage; hPGDS, hemopoietic PGD synthase; poly I:C, polyinosine-polycytidylic acid; TP, thromboxane A₂ receptor.

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activity of PGD₂ on eosinophils (26, 27). We and others have recently shown that CRTH2 agonists administered into the trachea led to translocation of eosinophils from the bloodstream into the airway (28–30), and that CRTH2-deficient mice exhibited decreased infiltration of eosinophils and other inflammatory cells during chronic allergic skin inflammation (31).

Infection with virus immediately activates the innate immune system via several pathways (32). dsRNA, synthesized during replication of RNA virus, is recognized by membrane or cytosolic receptors, such as TLR3 (33), retinoic-acid inducible gene-1, and melanoma differentiation-associated gene-5 (34, 35). In asthmatics, infection with RNA viruses leads to an abbreviated innate host defense response which allows the replication of virus and the eventual release, during cell necrosis, of large amounts of virus-derived dsRNA (36). The activation of these pattern-recognition receptors by dsRNA triggers the synthesis of type I IFNs and other cytokines/chemokines in airway epithelial cells, dendritic cells, and other immune cells (32, 37), however, little is known about its impact on the axis of lipid mediators and their receptors.

As reported herein, we developed murine models mimicking the enhanced asthmatic responses induced by viral infection. In these models, we used the intratracheal administration of dsRNA to simulate viral infection and showed that it worsened allergen-induced eosinophilic airway inflammation and airway hyperresponsiveness. In these experiments, we also establish that COX-2 is induced in the lungs of allergen-exposed animals and that PGD₂, synthesized via activation of COX-2, acting via the CRTH2 receptor, is the essential modulator of eosinophilic airway inflammation and bronchial hyperresponsiveness induced by dsRNA instillation.

Materials and Methods

Animals

Specific pathogen- and virus Ab-free (SPF/VAF) 8-wk-old, male Brown Norway (BN) rats (BN/CrCrJ) weighing between 230 and 250 g, and SPF/VAF, 8-wk-old, male BALB/c mice (BALB/cAnCrCrJ), weighing between 25 and 30 g, were purchased from Charles River Laboratories. SPF/VAF male CRTH2-deficient (BALB/c) mice (31) were bred in the Keio University Animal Research Facility. All animals were housed at the facility in bioBubble barrier units under positive pressure. The experimental protocol was reviewed and approved by the Laboratory Animal Care and Use Committee of Keio University School of Medicine.

Protocols for allergen exposure and dsRNA administration

Rats were actively sensitized on days 0 and 14 against OVA (albumin grade V from chicken egg; Sigma-Aldrich) by s.c. injection of 1 mg of OVA with 0.28 ml of alum solution (ImjectAlum; Pierce) consisting of 40 mg/ml Al(OH)₃ and 40 mg/ml Mg(OH)₂. *Bordetella pertussis* vaccine containing 6×10^9 heat-killed bacilli (Wako Pure Chemical) was also administered i.p. on day 0 (38, 39). On day 21, the rats were exposed to aerosols of 0.5% (w/v) OVA in PBS, or PBS alone generated with an ultrasonic nebulizer (NE-U17; Omron) for 30 min in an acrylic chamber, and 16 h after exposure to OVA/PBS, polyinosine-polycytidylic acid (poly I:C; 100 µg/animal, GE Healthcare Bio-Sciences), PGD₂ (0.1–1 nM/animal, Cayman Chemical), or the vehicle (100 µl of PBS) were sprayed intratracheally using a MicroSprayer aerosolizer (model IA-1C with FMJ-250 high-pressure syringe; Penn-Century) and a laryngoscope (LS-1: Small Animal Laryngoscope; Penn-Century) under anesthesia with i.p. 100 mg/kg ketamine and 10 mg/kg xylazine. In some experiments, prostanoid receptor antagonists, such as BW A868C (a DP1-specific antagonist, 1 mg/kg i.v.; Cayman Chemical), SQ29,548 (a thromboxane A₂ receptor (TP)-specific antagonist, 2.5 mg/kg i.v.; Cayman Chemical), and ramatroban (BAY-u3405; a dual receptor antagonist for CRTH2 and TP, 30 mg/kg per os; Bayer Yakuhin), were administered 1 h before the instillation of poly I:C.

Mice were actively sensitized against OVA by the s.c. injection of 15 µg of OVA and 25 µl of Imject Alum on days 0, 7, and 14. On days 21–24, the mice were exposed to aerosols of 1% (w/v) OVA in PBS, or PBS alone generated with an ultrasonic nebulizer for 20 min in an acrylic chamber, and 16 h after the final exposure to OVA/PBS on day 24, poly I:C (100

µg/animal) or the vehicle (PBS) were sprayed intratracheally with a MicroSprayer aerosolizer under anesthesia with i.p. 100 mg/kg ketamine and 10 mg/kg xylazine.

Bronchial responsiveness to methacholine

Bronchial responsiveness in rats was measured 24 h after the OVA challenge. The trachea was cannulated with a 14-gauge tube under anesthesia with i.p. 100 mg/kg ketamine and 10 mg/kg xylazine and the animals were quasiosinuously ventilated with a computer-controlled small-animal ventilator (flexiVent; SCIREQ), with a 9.2 ml/kg tidal volume set automatically depending on body weight, at 90 breaths/min and positive end-expiratory pressure of 3.0 cmH₂O. The airway resistance was measured with the same system by the forced oscillation technique after inhalation of five doses of aerosolized methacholine solution (0, 6.25, 12.5, 25.0, and 50.0 mg/ml in PBS; Sigma-Aldrich) for 10 s, with a tidal volume of 18.0 ml/kg at 50 breaths/min. Before each aerosol challenge, the animal was given two deep inspirations to standardize the volume history. After each methacholine challenge, the respiratory system resistance was recorded during tidal breathing every 10 s, and the peak resistance measured was used as the bronchoconstrictor response to that methacholine dose.

Bronchoalveolar lavage (BAL)

Rats and mice were sacrificed by an overdose of i.v. pentobarbital. The trachea was cannulated and the lungs were lavaged three times with PBS (pH 7.4) containing 0.6 mM EDTA (8 ml for rats, 1.4 ml for mice). Total cells in BAL fluid were counted, using a hemocytometer, and the cell types were identified on Diff-Quik-stained cytospin slides prepared with Auto Smear CF12D (Sakura Finetek). Two hundred cells in duplicate slides were counted in a blinded fashion.

Concentrations of rat IL-4, IL-12 p70, IL-13, IFN-γ, and CCL5/RANTES were measured, using ELISA purchased from BioSource International, and concentrations of CCL11/eotaxin-1 were measured with ELISA from R&D Systems according to the manufacturers' instructions. Cytokine levels in mouse BAL fluids were determined using a Bio-Plex suspension array system (Bio-Rad) according to the manufacturer's instructions.

Histological examination

After the completion of BAL, the chest was opened and the pulmonary circulation thoroughly flushed with PBS using a peristaltic pump at a flow rate of 5 ml/min, through a catheter inserted in the pulmonary artery. The lungs were removed and fixed in 4% (w/v) neutralized buffered paraformaldehyde (pH 7.4) at 4°C. The lung tissues were paraffin embedded and 4-µm sections were stained with Giemsa. A semiquantitative scoring system was used to grade the degree of eosinophil accumulation as previously reported (30). In brief, two separate investigators examined 10 bronchi in the lungs in a blinded manner, grading each bronchus from 0 (no eosinophils) to 4 (abundant eosinophilic infiltration) and the scores were averaged (0–4).

Immunofluorescence analysis of lung COX-2 expression

Immunofluorescence staining of COX-2 was performed on 4-µm-thick sections. After deparaffinization and Ag retrieval using a commercially available buffer (L.A.B. Solution; Polysciences), the slides were blocked with 10% normal goat serum, 2% BSA in PBS for 40 min, then incubated for 2 h with 1 µg/ml anti-COX-2 rabbit polyclonal Ab (Cayman Chemical) at room temperature. After wash with Image-iT FX signal enhancer (Invitrogen Life Technologies) for 30 min at room temperature, the slides were incubated for 30 min with 20 µg/ml Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen Life Technologies) at room temperature. The slides were mounted with ProLong Gold Antifade reagent (Invitrogen Life Technologies), then examined using a LSM-510 confocal laser-scanning microscope (Carl Zeiss).

Eicosanoid release from ex vivo lung culture or isolated alveolar macrophages

Lungs harvested 16 h after exposure to PBS or OVA were sliced (3-mm thick) in Ca²⁺ and Mg²⁺-free HBSS, and incubated for 2 h in RPMI 1640 medium at 37°C. The lungs were washed and incubated for 8 h in fresh medium, with or without 10 µg/ml poly I:C.

Alveolar macrophages were isolated from BAL fluid collected 16 h after exposure to PBS or OVA. BAL fluid was centrifuged at 400 × g for 5 min at 4°C, and the leukocytes in the pellet were resuspended and incubated in RPMI 1640 medium for 2 h at 37°C. The plates were washed twice to remove the nonadherent cells. Adherent cells were then incubated for 8 h in the fresh medium with or without 10 µg/ml poly I:C. The concentrations of leukotriene B₄, cysteinyl leukotrienes, thromboxane B₂, or PGD₂ in the

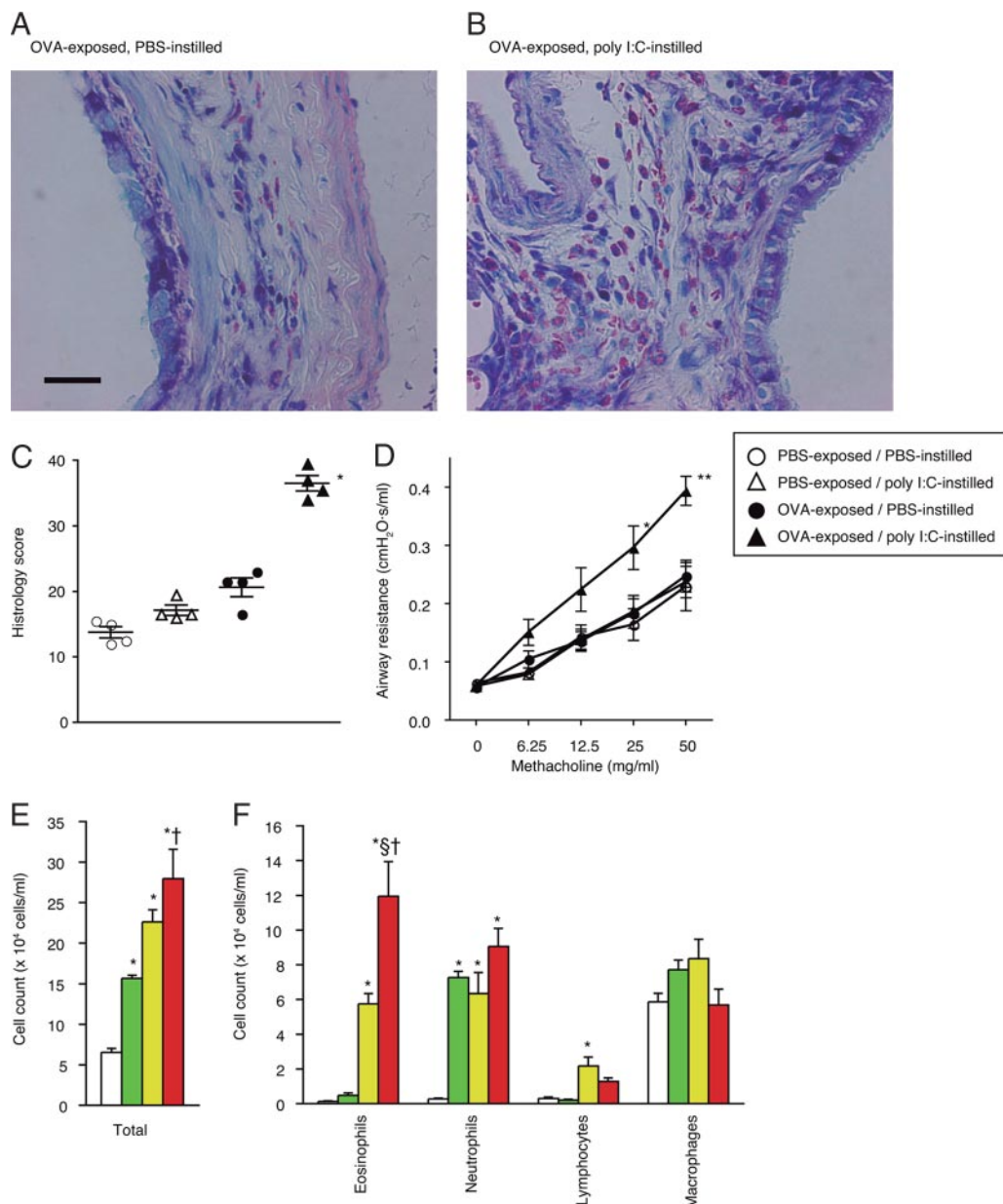


FIGURE 1. Poly I:C-induced enhancement of allergic airway inflammation and bronchial hyperresponsiveness in BN rats. PBS or poly I:C was instilled intratracheally 16 h after exposure to either PBS or OVA in sensitized rats. Eosinophil infiltration in the bronchial wall of OVA-exposed/PBS-instilled (A) and OVA-exposed/poly I:C-instilled animals (B). Tissue sections were stained with Giemsa. Bar, 100 μ m. C, Semiquantitative scoring of airway histology (eosinophil accumulation in the peribronchial area). Mean values $\pm$ SEM ($n = 4$ each); *, $p < 0.001$ vs OVA-exposed/PBS-instilled animals. D, Concentration-response curves of airway resistance to inhaled methacholine. Mean values $\pm$ SEM ($n = 9 - 13$); *, $p < 0.01$, **, $p < 0.001$ vs OVA-exposed/PBS-instilled animals. (E) Total and (F) differential cell counts of leukocytes in BAL fluid. PBS-exposed/PBS-instilled (white column; $n = 8$), PBS-exposed/poly I:C-instilled (green column; $n = 4$), OVA-exposed/PBS-instilled (yellow column; $n = 6$), OVA-exposed/poly I:C-instilled (red column; $n = 5$) animals; *, $p < 0.001$ vs PBS-exposed/PBS-instilled group; §, $p < 0.001$ vs OVA-exposed/PBS-instilled animals; †, $p < 0.001$ vs PBS-exposed/poly I:C-instilled group.

supernatant of lung slices or alveolar macrophages were measured using ELISA (Cayman Chemical). In some experiments, the lung slices or alveolar macrophages were pre- and coincubated with 1 μ M NS-398, a selective COX-2 inhibitor (Taisho-Toyama Pharmaceutical).

Quantitative RT-PCR

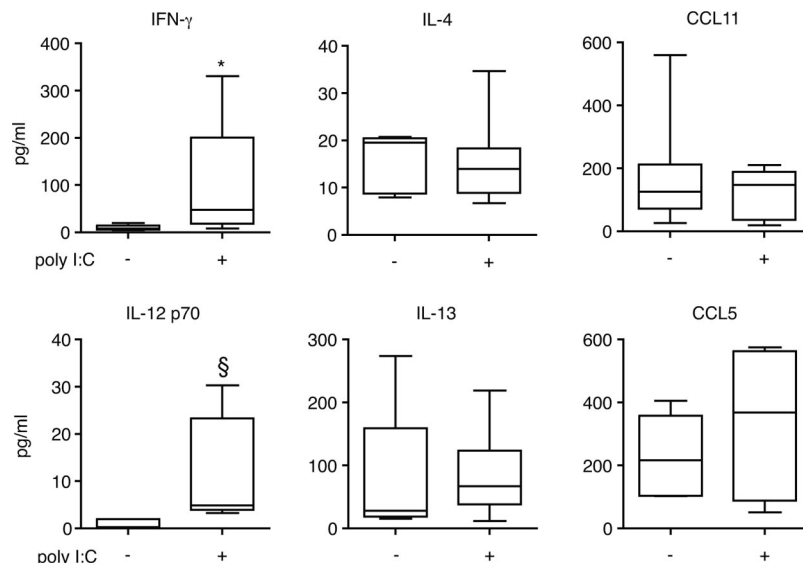
Isolated alveolar macrophages recovered 16 h after PBS or OVA exposure were incubated for 8 h, with or without 10 μ g/ml poly I:C, as described earlier. Total RNA was extracted using the RNeasy Mini kit (Qiagen). The mRNA levels of COX-2 and hemopoietic PGD synthase (hPGDS) were measured with quantitative RT-PCR. Reverse transcription was performed using SuperScript II (Invitrogen Life Technologies) followed by quantitative PCR amplification using the TaqMan method (ABI PRISM 7000; Ap-

plied Biosystems). Premixed PCR primers and TaqMan probes for rat COX-2, hPGDS, and GAPDH (Assay-on-Demand) were obtained from Applied Biosystems. The conditions for PCR were as follows: 1 cycle of 95°C for 9 min, 50 cycles of 95°C for 30 s and 60°C for 1 min, and 1 cycle of 72°C for 5 min.

Analysis of poly I:C uptake in alveolar macrophages

Poly I:C was labeled with fluorescein using the FastTag FL kit (Vector Laboratories), according to the manufacturer's instructions, as previously reported (40). Isolated alveolar macrophages from rats sacrificed 16 h after PBS or OVA exposure were incubated with 100 μ g/ml unlabeled or fluorescein-labeled poly I:C, or PBS in RPMI 1640 containing 10% FBS for 30 min at 37°C. After three washes with ice-cold PBS, the alveolar macrophages were

FIGURE 2. Concentrations of cytokines and chemokines in the BAL fluid of OVA-exposed/PBS-instilled and OVA-exposed/poly I:C-instilled rats ($n = 5-12$). The box-whisker plots show the 25th and 75th percentiles, the median (horizontal line within the box), and the 10th and 90th percentiles (whiskers); *, $p < 0.05$, §, $p < 0.005$, vs OVA-exposed/PBS-instilled animals.



fixed with 10% paraformaldehyde at 4°C for 30 min, permeabilized with 0.1% saponin in 2% BSA-PBS at 4°C for 30 min, then stained with 20 $\mu\text{g/ml}$ Alexa Fluor 488-conjugated rabbit anti-fluorescein IgG (Invitrogen Life Technologies) for 1 h at 4°C. The labeled cells were analyzed using a FACScan flow cytometer (BD Biosciences).

Statistical analyses

Numbers of leukocytes in BAL fluid, production of eicosanoid from ex vivo organ cultures or isolated alveolar macrophages, and mRNA levels of PGD_2 synthetic enzymes were tested with one-way ANOVA, followed by Bonferroni/Dunn procedure as a post-hoc test. Concentrations of cytokines and chemokines were compared by two-tailed Mann-Whitney U test. Bronchial responsiveness to methacholine was analyzed with two-way repeated-measures ANOVA, followed by Bonferroni/Dunn procedure as a post-hoc test. Statistical analyses were performed, using GraphPad Prism 4.0c (GraphPad Software) and StatView 5.0 (SAS Institute). A p value < 0.05 was considered statistically significant.

Results

BN rats, sensitized with i.p. injection of OVA/alum followed 21 days later by a single exposure to aerosolized OVA at a 0.5% concentration, developed minimal airway inflammation with eo-

sinophil infiltration into the bronchial wall (Fig. 1A), and similar bronchial responsiveness to methacholine compared with OVA-sensitized, PBS-exposed animals. The synthetic dsRNA, poly I:C, instilled intratracheally 16 h after the exposure to OVA, increased the number of eosinophils in the subepithelial layer of the bronchial wall significantly (Fig. 1B). Semiquantitative evaluation of the airway histology confirmed the enhanced eosinophilic inflammation in the

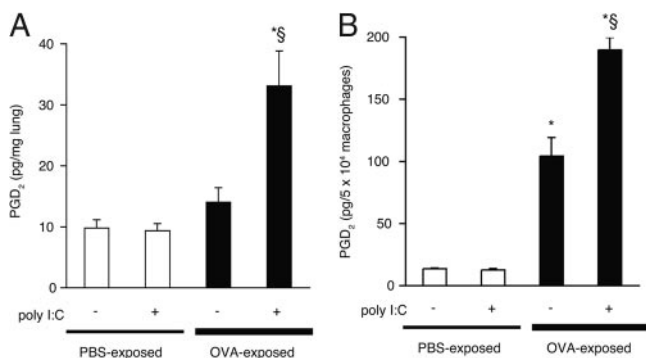


FIGURE 3. A, Ex vivo PGD_2 production in the lungs of PBS-exposed ($\square$, $n = 8$) or OVA-exposed ($\blacksquare$, $n = 12-16$) rats, cultured for 8 h in the presence or absence of 10 $\mu\text{g/ml}$ poly I:C. Mean values $\pm$ SEM; *, $p < 0.001$ vs lungs isolated from PBS-exposed animals; §, $p < 0.005$ vs lungs isolated from OVA-exposed animals cultured without poly I:C. B, PGD_2 production from alveolar macrophages isolated from PBS- or OVA-exposed rats ($n = 4$ each) cultured for 8 h in the presence or absence of 10 $\mu\text{g/ml}$ poly I:C. Mean values $\pm$ SEM; *, $p < 0.001$ vs alveolar macrophages isolated from PBS-exposed animals; §, $p < 0.001$ vs alveolar macrophages isolated from OVA-exposed animals cultured without poly I:C.

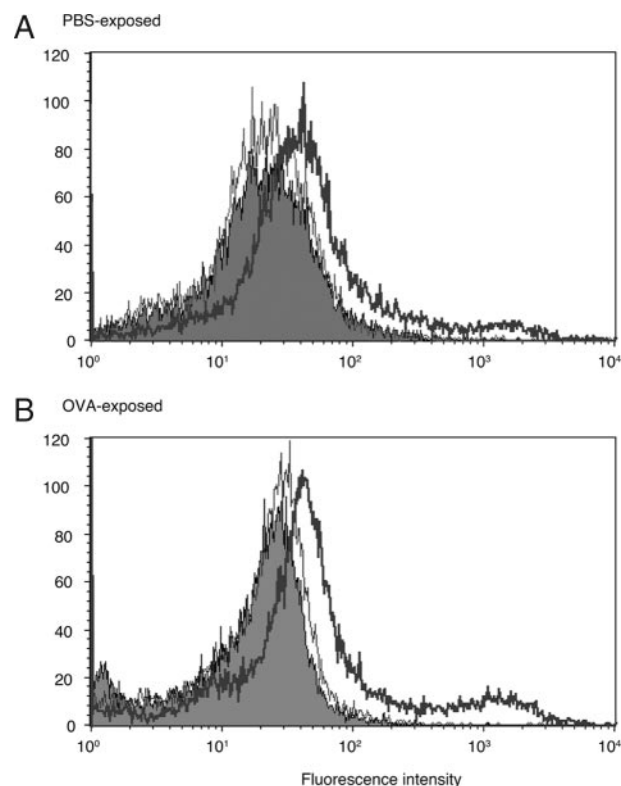


FIGURE 4. Flow cytometric analysis of fluorescein-labeled poly I:C-uptake in alveolar macrophages isolated from PBS-exposed rats (A) and OVA-exposed rats (B). Shaded area represents fluorescence intensity of PBS-treated macrophages, thin lines represent fluorescence intensity of unlabeled poly I:C-treated macrophages, and bold lines represent fluorescence intensity of fluorescein-labeled poly I:C-treated macrophages. Results of a single representative experiment (of four) are shown.

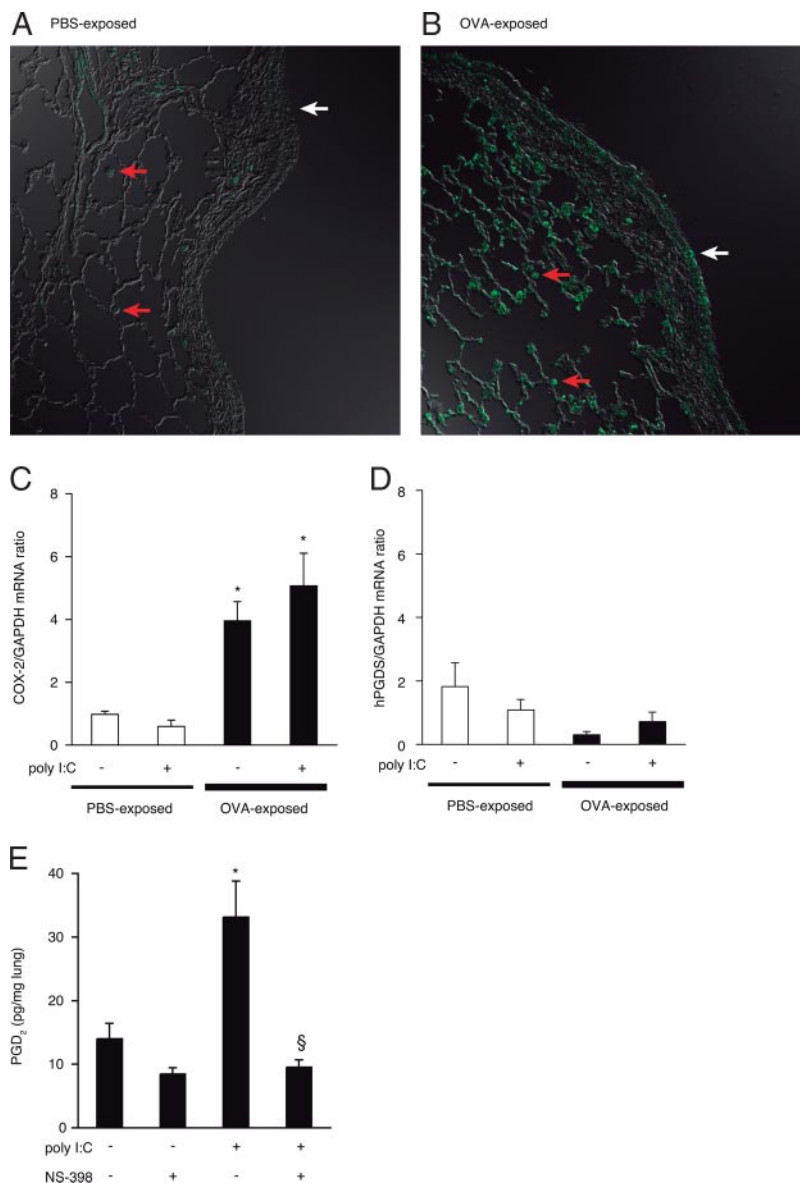


FIGURE 5. COX-2 expression and COX-2-dependent PGD₂ production in PBS/OVA-exposed rat lungs. Immunofluorescence analysis of COX-2 expression in the lungs exposed to PBS (A) or OVA (B). COX-2 is expressed mostly in alveolar macrophages (red arrows), and in some bronchial epithelial cells (white arrows). Quantitative RT-PCR of COX-2 mRNA (C) and hPGDS mRNA (D) in alveolar macrophages isolated from rats exposed to PBS (□, $n = 4$) or OVA (■, $n = 7$) cultured with or without 10 μ g/ml poly I:C. Mean values $\pm$ SEM; *, $p < 0.005$, vs alveolar macrophages isolated from PBS-exposed animals. E, Effects of a COX-2-specific inhibitor, NS-398, 1 μ M, on the production of PGD₂ in ex vivo lung cultures from OVA-exposed rats ($n = 4$ –8). Mean values $\pm$ SEM; *, $p < 0.005$ vs lungs cultured without poly I:C; §, $p < 0.005$ vs lungs cultured with poly I:C in absence of NS-398.

peribronchial space of OVA-exposed/poly I:C-instilled animals (Fig. 1C; $n = 4$, $p < 0.001$ compared with OVA-exposed/PBS-instilled animals). Bronchial responsiveness to methacholine was also enhanced in OVA-exposed/poly I:C-instilled animals compared with OVA-exposed/PBS-instilled animals ($p < 0.001$, Fig. 1D). The number of eosinophils in the BAL fluid was greater in OVA-exposed/poly I:C-instilled animals (11.9 ± 2.0 cells/ml, $n = 5$) than in OVA-exposed/PBS-instilled animals (5.8 ± 0.6 cells/ml, $n = 6$, $p < 0.05$, Fig. 1, E and F). The intratracheal administration of poly I:C in PBS-exposed animals caused a significant increase in the number of neutrophils in BAL fluid, though no airway eosinophilia or bronchial hyperresponsiveness was observed (Fig. 1, C–F). The intratracheal instillation of ssRNA, polycytidylic acid, or dsDNA, polydeoxyinosine-polydeoxycytidylic acid, in OVA-exposed rats had no effect on histological architecture or the differential cell counts in BAL fluid (data not shown).

To clarify the mechanisms by which the intratracheal instillation of poly I:C exacerbated the allergen-induced airway eosinophilia and bronchial hyperresponsiveness, we analyzed the profile of cytokines, chemokines, and lipid mediators in the lungs. The concentrations of Th1 cytokines, IFN- γ and IL-12 p70, were significantly higher in the BAL fluid of OVA-ex-

posed/poly I:C-instilled than in the fluid of OVA-exposed/PBS-instilled animals ($n = 5$ –12, $p < 0.005$ –0.05, Fig. 2). The effect of poly I:C instillation was less significant in PBS-exposed animals ($n = 6$ –12); the level of IFN- γ was 2.1 pg/ml (median, interquartile range, 1.1–7.4 pg/ml) in the PBS/PBS group and 8.9 pg/ml (1.2–20.7 pg/ml) in the PBS/poly I:C group, while IL-12 p70 levels were 4.5 pg/ml (2.6–9.5 pg/ml) in the PBS/PBS group and 4.3 pg/ml (3.5–5.9 pg/ml) in the PBS/poly I:C group. In contrast to Th1 cytokines, there was no difference in the concentrations of Th2 cytokines, such as IL-4 and IL-13, or eosinophilic CC chemokines, such as CCL5/RANTES and CCL11/eotaxin-1 in the BAL fluid between OVA-exposed/PBS-instilled and OVA-exposed/poly I:C-instilled animals (Fig. 2).

We next analyzed the profile of lipid mediators synthesized in the lungs in response to poly I:C using an ex vivo organ culture system. Lungs harvested 16 h after the final exposure to PBS or OVA from sensitized rats were incubated for 8 h in presence vs absence of 10 μ g/ml poly I:C and the concentrations of lipid mediators in the culture supernatant were measured. Poly I:C increased the production of PGD₂ by the lungs pre-exposed to OVA significantly, though not by the lungs pre-exposed to PBS

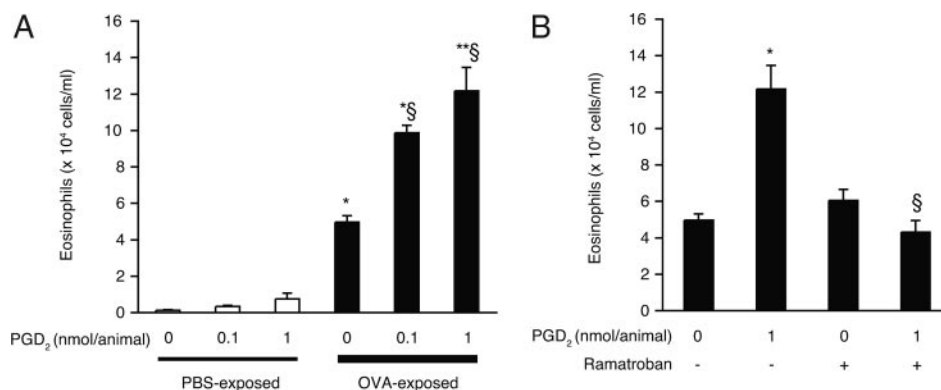


FIGURE 6. Role of CRTH2 receptor in PGD₂-induced accumulation of eosinophils in the rat airways. **A**, PGD₂, 0.1–1 nM/animal, administered intratracheally increased significantly the number of eosinophils in BAL fluid from OVA-exposed rats ($n = 3$ –17), but not from PBS-exposed rats ($n = 3$ –8). Mean values + SEM; *, $p < 0.005$, **, $p < 0.001$ vs PBS-exposed/PBS-instilled rats, §, $p < 0.001$ vs OVA-exposed/PBS-instilled rats. **B**, PGD₂, 1 nM/animal, induced BAL eosinophilia in OVA-exposed rats pretreated with or without ramatroban (30 mg/kg), a CRTH2/TP antagonist; $n = 4$ –17. Mean values + SEM. *, $p < 0.001$ vs OVA-exposed/PBS-instilled rats pretreated with the vehicle, §, $p < 0.001$ vs OVA-exposed/PGD₂-instilled rats pretreated with the vehicle.

(Fig. 3A). Alveolar macrophages isolated from OVA-exposed lungs demonstrated greater release of PGD₂ without a challenge stimulus than did macrophages isolated from PBS-exposed lungs. Coincubation with poly I:C (10 μ g/ml) resulted in a fur-

ther increase in the synthesis of PGD₂ in alveolar macrophages isolated from OVA-exposed lungs only (Fig. 3B). No effect of poly I:C was observed on the synthesis of leukotriene B₄, cysteinyl leukotrienes, or thromboxanes (data not shown).

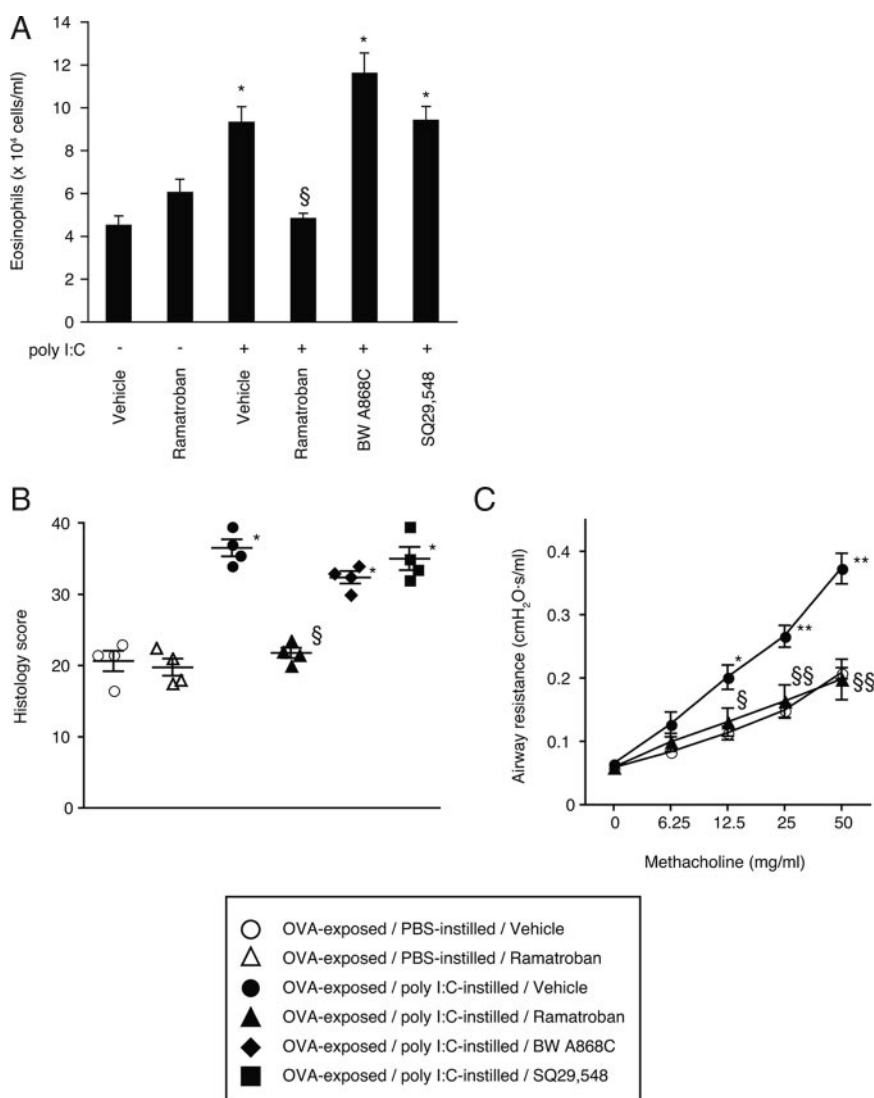
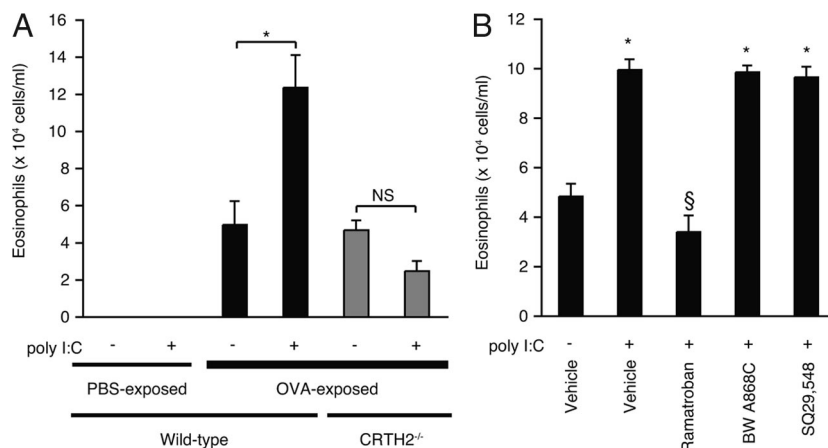


FIGURE 7. Role of CRTH2 receptor in poly I:C-induced exacerbation of eosinophilic airway inflammation in OVA-exposed rats. **A**, Number of eosinophils in BAL fluid from OVA-exposed, PBS-, or poly I:C (100 μ g/animal)-instilled rats. The rats were pretreated with ramatroban, a 30 mg/kg CRTH2/TP antagonist, or BW A868C, a 1 mg/kg DP1 antagonist, or SQ29548, a 2.5 mg/kg TP antagonist; $n = 4$ –11. Mean values + SEM. *, $p < 0.001$ vs OVA-exposed/PBS-instilled rats; §, $p < 0.001$ vs OVA-exposed/poly I:C-instilled rats pretreated with the vehicle. **B**, Semiquantitative scoring of eosinophil accumulation in the peribronchial area. Mean values $\pm$ SEM ($n = 4$ each); *, $p < 0.001$ vs OVA-exposed/PBS-instilled rats pretreated with the vehicle; §, $p < 0.001$ vs OVA-exposed/poly I:C-instilled rats pretreated with the vehicle. **C**, Bronchial responsiveness to methacholine in OVA-exposed/PBS-instilled rats ($n = 7$), OVA-exposed/poly I:C-instilled rats pretreated with vehicle ($n = 8$) or ramatroban ($n = 9$). Mean values $\pm$ SEM. *, $p < 0.01$, **, $p < 0.001$ vs OVA-exposed/PBS-instilled rats pretreated with the vehicle; §, $p < 0.01$, §§, $p < 0.001$ vs OVA-exposed/poly I:C-instilled rats pretreated with the vehicle.

FIGURE 8. Poly I:C-induced exacerbation of eosinophilic airway inflammation in wild-type and CRTH2-deficient (CRTH2^{-/-}) mice. **A**, Number of eosinophils in BAL fluid. Poly I:C, 100 μ g/animal, was instilled intratracheally in wild-type or CRTH2^{-/-} mice exposed to PBS or OVA; $n = 4$ –10. Mean values $\pm$ SEM; *, $p < 0.001$. NS: not significant. **B**, Number of eosinophils in BAL fluid from OVA-exposed, poly I:C (100 μ g/animal)-instilled wild-type mice. Mice were pretreated with the vehicle, or 30 mg/kg ramatroban, or 1 mg/kg BW A868C, or 2.5 mg/kg SQ29548; $n = 4$ –9. Mean values $\pm$ SEM; *, $p < 0.001$ vs OVA-exposed/PBS-instilled mice, §, $p < 0.001$ vs OVA-exposed/poly I:C-instilled mice pretreated with the vehicle.



Because there was a significant difference in the amount of PGD₂ produced between alveolar macrophages isolated from PBS- and OVA-exposed lungs, we examined the capacity of these cells to take up exogenously administered, fluorescein-labeled poly I:C. There was no difference in poly I:C-uptake between alveolar macrophages from PBS- vs OVA-exposed animals (Fig. 4).

We then hypothesized that there was an increase in the expression or activity of the enzymes in the OVA-exposed lungs required to synthesize PGD₂. Using immunofluorescence analysis, we observed the presence of prominent COX-2 immunoreactivity in OVA-exposed lungs, particularly in alveolar macrophages, however, not in PBS-exposed lungs (Fig. 4). In addition to increased amounts of protein product, experiments with quantitative RT-PCR confirmed that the expression of COX-2 mRNA in isolated alveolar macrophages was higher in the OVA-exposed as compared with control lungs (Fig. 5C). In contrast, the levels of hemopoietic PGD synthase (hPGDS) mRNA (Fig. 5D) were similar in both groups. Treatment with the relatively COX-2-specific inhibitor, NS-398, 1 μ M, which completely blocked the COX-2-, but not COX-1-, dependent synthesis of PGE₂ in cultured lung epithelial cells (41), significantly decreased the poly I:C-stimulated synthesis of PGD₂ in ex vivo lung cultures from OVA-exposed animals (Fig. 5E), or in alveolar macrophages (data not shown).

We next examined whether PGD₂ was capable of recruiting eosinophils to the site of inflammation in the airways. As we have previously reported (30), intratracheal instillation of PGD₂ in PBS-exposed rats did not increase the number of eosinophils in BAL fluid. In contrast, PGD₂, in a dose of 0.1–1 nM/animal, produced a significant increase in the number of eosinophils in BAL fluid of OVA-exposed animals (Fig. 6A). Pretreatment with ramatroban, a dual receptor antagonist for CRTH2 and TP receptors, suppressed the number of eosinophils in BAL fluid significantly in OVA-exposed, PGD₂-instilled animals (Fig. 6B). This observation suggested that CRTH2 is essential for the accumulation of eosinophils induced by PGD₂ in the preinflamed airways in vivo. We next used this set of experimental interventions to determine the role of CRTH2 in the poly I:C-induced enhancement of eosinophilic airway inflammation. Ramatroban, but not BW A868C, a DP1-specific antagonist, or SQ29,548, a TP-specific antagonist, markedly suppressed the number of eosinophils in BAL fluid (Fig. 7A) or in the bronchial wall (Fig. 7B) in OVA-exposed, poly I:C-instilled animals. Furthermore, bronchial hyperresponsiveness to methacholine in OVA-exposed/poly I:C-instilled animals was decreased by pretreatment with ramatroban to the levels of PBS-exposed animals (Fig. 7C).

To further examine the effects of poly I:C on eosinophilic inflammation of the airways, we induced this condition by repeated

exposures of previously sensitized BALB/c mice to aerosolized OVA for 4 days. As in BN rats, the intratracheal instillation of 100 μ g/animal of poly I:C had no effects on the number of eosinophils in BAL fluid in PBS-exposed mice, though there were significantly increased numbers of eosinophils in OVA-exposed animals (Fig. 8A). In this model, we examined the role of PGD₂ and CRTH2 in poly I:C-induced exacerbation of airway eosinophilia, using genetically engineered CRTH2-deficient mice (31). It is noteworthy that there was no difference in the levels of allergen-induced airway eosinophilia between CRTH2^{-/-} and wild-type mice, though the CRTH2^{-/-} mice completely lacked the response to poly I:C on airway eosinophil accumulation (Fig. 8A). CRTH2 deficiency had no effects on the number of neutrophils or lymphocytes. The decreased airway eosinophilia in CRTH2-deficient mice after OVA/poly I:C-exposure cannot be attributed to the lack of Th2 cytokine responses in the airways. IL-5 levels in BAL fluids were not significantly different between wild-type mice (median 19.6 pg/ml, interquartile range 14.7–29.9 pg/ml, $n = 8$) and CRTH2-deficient mice (31.1 pg/ml, 11.5–103 pg/ml, $n = 7$), nor were IL-13 levels (28.8 (24.7–39.4) pg/ml in wild-type mice and 54.1 (7.2–64.6) pg/ml in CRTH2-deficient mice). Furthermore, pretreatment with ramatroban, but not with BW A868C or SQ29,548, prevented the BAL eosinophilia induced by poly I:C in wild-type mice (Fig. 8B), supporting the role of the PGD₂/CRTH2 pathway on the dsRNA-induced exacerbation of allergic airway inflammation.

Discussion

In the present study, we developed two experimental models that mimic the pathological and physiological changes observed in the airways of asthmatic patients during acute exacerbations induced by viral infections, using different animals and protocols of allergen exposure. DsRNA instilled in the airway, which, alone, did not cause the accumulation of eosinophils, exacerbated the eosinophilic inflammation in the lungs of allergen-exposed rats and mice, and increased the bronchial responsiveness to methacholine in rats. We then established the essential role played by the COX-2/PGD₂/CRTH2 pathway in the dsRNA-induced exacerbation of airway inflammation and/or bronchial hyperresponsiveness in both models. The expression of COX-2 induced by allergen exposure increased the synthesis of PGD₂ in response to dsRNA, which recruits eosinophils into the airways through CRTH2, a PGD₂ receptor.

We have shown, in this study, that 1) the administration of poly I:C into the airways increased the synthesis of PGD₂ in OVA-exposed lungs only (Fig. 3), 2) intratracheally instilled PGD₂ recruited eosinophils into the airways of OVA-exposed rats (Fig. 6) and mice (data not shown), 3) the pharmacological blockade of

CRTH2, a PGD₂ receptor, prevented the increase in eosinophil accumulation and/or bronchial hyperresponsiveness induced by the administration of poly I:C in rats (Fig. 7) and mice (Fig. 8B), and 4) CRTH2 gene-deficient mice had similar responses to allergen exposure as wild-type mice, though did not show the exacerbation of eosinophilic inflammation induced by poly I:C that was observed in wild-type mice (Fig. 7A). All of these observations strongly indicate that PGD₂ and its receptor CRTH2 is the essential system for the exacerbation of eosinophilic airway inflammation induced by dsRNA.

We previously reported that exposure to an allergen increases the expression of COX-2 in the lungs of a sensitized guinea pig, and increased the synthesis of PGD₂ and PGE₂, but not prostacyclin, thromboxanes, or leukotrienes (19), corresponding closely to the profile of eicosanoid synthesis observed in the OVA-exposed, poly I:C-stimulated rat lungs in the present study. Therefore, we analyzed the expression of COX-2 in the lungs by immunofluorescence and found that the immunoreactivity of COX-2 was up-regulated in alveolar macrophages and bronchial epithelial cells of OVA-exposed rats. The induction of COX-2 in alveolar macrophages was also confirmed at the levels of mRNA, as well as by the pharmacological blockade of PGD₂ synthesis by NS-398, a COX-2-specific inhibitor. In contrast, there was no significant difference between PBS- or OVA-exposed macrophages, in the expression of hPGDS, the dominant isoform of PGDS in the airways, or in the capacity for macrophages to take up dsRNA, suggesting that the increased synthesis of PGD₂ in OVA-exposed, poly I:C-stimulated lungs was mostly dependent on the induction of COX-2. Taha et al. (42) observed that the induced sputum in patients with asthma contained a significantly higher ratio of COX-2 immunopositive cells, mainly eosinophils, neutrophils, and macrophages, than in control subjects. According to their report, 16.0% of macrophages were immunostained with COX-2 in controls, vs 53.1% in asthmatics. It has also been reported that viral infection or treatment with poly I:C induces the expression of COX-2 in macrophages and increases the production of PGs (43). The role of mast cells, another major source of PGD₂ in the airways (44), remains to be defined. It is possible that the sensitization protocol in our system, which used OVA with alum, has made it difficult to evaluate the role of mast cells as previously reported (45). Future studies using different sensitization protocols or mast cell-deficient mice such as *W^{sh}/W^{sh}* mice may clarify the role of mast cell-derived PGD₂ in this system.

PGD₂ transduces its biological actions through stimulation of the DP1 and CRTH2 (DP2), two G protein-coupled receptors expressed on the surface of eosinophils (15, 16). CRTH2 has been shown to mediate the PGD₂-induced mobilization of eosinophils in vitro (15, 16, 22) and in vivo (28–30, 46). In a previous study, we found that intratracheal PGD₂ induced a marked influx of eosinophils into the airways of rats pretreated with i.v. IL-5, but not of untreated rats (30). In the experiments reported herein, we found that PGD₂ recruited eosinophils into the airspace of allergen-exposed animals; there were, however, several differences in the responses to PGD₂ between the IL-5-pretreatment and the OVA-sensitization/exposure models. First, a lower dose of PGD₂ (0.1–1 nM/animal) was needed to induce an equivalent amount of airway eosinophilia in the OVA-exposed than in the IL-5-pretreated model (10–100 nM/animal). We, however, still have to be cautious about the interpretation of this data, because endogenous levels of PGD₂ may be further lower judging from the data in ex vivo organ culture experiments (Fig. 3A). Second, the eosinophilia induced by PGD₂ persisted for a longer period of time in the OVA-sensitization/exposure model. Although BAL eosinophilia peaked at 2 h after PGD₂ instillation and returned to baseline within 8 h in

the IL-5-pretreated rats, it remained elevated for >8 h in the OVA-exposed animals. These observations suggest that dsRNA induces the expression of another molecule(s), such as adhesion molecules (47), which augment and/or maintain the airway eosinophilia by interacting with PGD₂.

In conclusion, allergen-induced inflammation led to an increased susceptibility of the airways to dsRNA and increased their likelihood to develop eosinophilic inflammation and bronchial hyperresponsiveness via the activation of the COX-2/PGD₂/CRTH2 pathway. The responsiveness to dsRNA that we observed appears enhanced in the inflamed airways, because 1) they have a higher capacity of producing PGD₂ in response to dsRNA due to the increased expression of COX-2 and 2) eosinophils are more likely to be recruited into the inflamed airways in response to PGD₂ through a CRTH2 receptor. The role of the CRTH2 receptor on the pathogenesis of allergic inflammation remains controversial. Although CRTH2 receptor antagonists have been found effective to suppress inflammation (48), CRTH2-deficient mice developed more severe inflammation of the airways compared with wild-type mice (49). However, in our model of virus infection-induced airway and airspace inflammation, pharmacological blockade or genetic deletion of CRTH2 each successfully suppressed airway inflammation.

COX-2 inhibitors were not only capable of suppressing the synthesis of PGD₂, but also that of PGE₂ in the airways of our rat models (data not shown). PGE₂ or forskolin, which elevates intracellular cAMP levels, has been demonstrated to induce the expression of 15-lipoxygenase and promote the lipoxin formation, which is essential for the resolution of inflammation (50). Thus, the COX-2 inhibitors block the synthesis of both pro- and anti-inflammatory mediators, and may not be beneficial to control airway inflammation (51). In contrast, the blockade of CRTH2, which is coupled with G_iα protein and suppresses intracellular cAMP levels, is less likely to compromise 15-lipoxygenase expression or lipoxin synthesis. It is tempting to speculate that CRTH2 receptor antagonism might be useful as a preventive or therapeutic agent in acute exacerbations of asthma.

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Disclosures

The authors have no financial conflict of interest.

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