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Prostaglandin D_2-induced eosinophilic airway inflammation is mediated by CRTH2 receptor

Yoshiki Shiraishi[*§], Koichiro Asano[*§], Takeshi Nakajima[*§], Tsuyoshi Oguma[*], Yusuke Suzuki[*], Tetsuya Shiomi[*], Koichi Sayama[*], Kyoko Niimi[*§], Misa Wakaki[*§], Junko Kagyo[*§], Eiji Ikeda[†], Hiroyuki Hirai[¶], Kazuhiro Yamaguchi[*§], and Akitoshi Ishizaka[*§]

[*]Division of Pulmonary Medicine, Department of Medicine (Y.S., K.A., T.N., T.O., Y.S., T.S., K.S., K.N., M.W., J.K., K.Y., A.I.), [†]Department of Pathology (E.I.), [§]Pfizer-Keio Research Laboratories, Shinanomachi Research Park (Y.S., K.A., T.N., K.N., M.W., J.K., K.Y., A.I.), Keio University School of Medicine, Tokyo, Japan
[¶]R&D Center, BML Laboratories (H.H.), Saitama, Japan

Running Title

PGD_2-induced airway eosinophilia and CRTH2 receptor

Address correspondence to:
Koichiro Asano, M. D.
Division of Pulmonary Medicine
Department of Medicine
Keio University School of Medicine
35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan
Phone: +81-3-3353-1211
FAX: +81-3-3353-2502
E-mail: ko-asano@qa2.so-net.ne.jp

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Abbreviations: PGD_2, prostaglandin D_2; IL-5, interleukin-5; DK-PGD_2, 13,14-dihydro-15-keto-PGD_2; MK-PGD_2, 11-deoxy-11-methylene-15-keto-PGD_2; BAL, ronchoalveolar lavage; TP, thromboxane A_2 receptor; BW 245C, [5-(6-carboxyhexyl)-1-(3-cyclohexyl-3-hydroxypropyl)-hydantoin; I-BOP, [1S-1α,2β(5Z),3α(1E,3R*),4α)]-7-[3-(3-hydroxy-4-(4'-iodophenoxy)-1-butenyl)-7-oxa bicyclo-[2.2.1]heptan-2-yl]-5-heptenoic acid: BW A868C, 3-benzyl-5-(6-carboxyhexyl)-1-(2-cyclohexy-2-hydroxyethylamino)-hydantoin;

SQ29,548, 5-heptenoic, 7-[3-[[2-[(phenylamino)carbonyl]hydrazino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-[1S-[1alpha ,2alpha (Z),3alpha ,4alpha]]

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Abstract

Mast cell-derived prostaglandin D_2 (PGD_2) is one of the essential modulators of eosinophilic airway inflammation in asthma and allergic rhinitis. Two G protein-coupled receptors for PGD_2, DP and CRTH2, are both expressed on the surface of eosinophils, and CRTH2 has been demonstrated to mediate PGD_2-induced eosinophil mobilization *in vitro*. However, it has not yet been determined whether PGD_2 and its receptors mediate *in vivo* eosinophil trafficking into the airways or other organs. We demonstrated that intratracheal administration of PGD_2 in rats pretreated with systemic interleukin-5 (IL-5) injection induced marked airway eosinophilia, determined by the differential counts of cells in bronchoalveolar lavage (BAL) fluid and lung histology, within 2 hours. Systemic IL-5 alone significantly increased the number of eosinophils in the peripheral blood, but showed no effect on airway eosinophilia. Three CRTH2-specific agonists (DK-PGD_2, MK-PGD_2, and indomethacin) demonstrated equivalent induction of BAL eosinophilia to that of PGD_2, but a DP agonist (BW 245C) or a TP agonist (I-BOP) showed no effect. PGD_2- or CRTH2 agonist-induced BAL eosinophilia was almost completely inhibited by pretreatment with a CRTH2/TP antagonist, ramatroban (BAY u-3405), while a TP-specific antagonist, SQ29,548, or a DP-specific antagonist, BW A868C, did not inhibit the effects of PGD_2. These results suggest that CRTH2 plays a significant role in the eosinophil trafficking from the bloodstream into the airways in PGD_2-related airway inflammation.

Introduction

Lipid mediators such as prostaglandins, leukotrienes, and platelet activating factor are essential modulators of allergic airway inflammation in asthma and allergic rhinitis. Several recent studies demonstrated that prostaglandin D_2 (PGD_2), which is the major cyclooxygenase metabolite released from activated mast cells, is also essential for the pathogenesis of eosinophilic airway inflammation (O'sullivan et al., 1998; Bochenek et al., 2004). PGD_2 is released during the early and late asthmatic responses following allergen exposure in patients with asthma (O'sullivan et al., 1998; Bochenek et al., 2004), and has been demonstrated to be a potent chemoattractant for eosinophils *in vitro* (Hirai et al., 2001; Monneret et al., 2001) and *in vivo* (Emery et al., 1989; Woodward et al., 1990). We previously reported that cyclooxygenase-2 inhibitors, which abolished PGD_2 synthesis in the lungs of allergen-sensitized and exposed guinea pigs, attenuated eosinophil accumulation in the airways (Oguma et al., 2002). Other researchers reported that overexpression of PGD_2 synthase or inhalation of aerosolized PGD_2 enhanced eosinophilic and lymphocytic airway inflammation in mice following allergen exposure (Fujitani et al., 2002; Honda et al., 2003).

The bioactivity of PGD_2 is mediated by two G protein-coupled receptors, DP and CRTH2, both of which are expressed on the surface of eosinophils (Gervais et al., 2001). DP receptor expression in the lungs is upregulated in allergic inflammation in mice (Matsuoka et al., 2000; Fujitani et al., 2002), and disruption of the DP gene abolished the accumulation of eosinophils and lymphocytes in a murine model of asthma (Matsuoka et al., 2000). DP receptor antagonists, such as S-5751 and BW A868C, also reduced the number of eosinophils in allergen-induced upper and lower and airway inflammation in guinea pigs (Arimura et al., 2001). However, a DP-specific agonist,

BW 245C, did not replicate the effects of PGD_2 on eosinophil mobilization *in vitro* (Hirai et al., 2001; Monneret et al., 2001) and *in vivo* (Woodward et al., 1990), suggesting that PGD_2-induced eosinophil chemotaxis is mediated by a receptor other than DP.

A second PGD_2 receptor, CRTH2, which has been identified as a molecule preferentially expressed on Th2 lymphocytes, eosinophils, and basophils, has been demonstrated to transduce direct chemotactic or chemokinetic activity of PGD_2 for eosinophils (Nagata et al., 1999a; Nagata et al., 1999b; Hirai et al., 2001; Monneret et al., 2001). A CRTH2-specific agonist, 13,14-dihydro-15-keto-PGD_2 (DK-PGD_2), enhances eosinophil migration and activation as potently as PGD_2 *in vitro* (Gervais et al., 2001; Hirai et al., 2001; Monneret et al., 2001). Therefore, it is likely that CRTH2, at least partially, mediates PGD_2-dependent eosinophil migration to inflammatory sites. However, there is no evidence suggesting that CRTH2-mediated signals are important *in vivo* for the accumulation of eosinophils in local organs such as the airways. We thus examined the role of the CRTH2 receptor in PGD_2-induced airway eosinophilic inflammation.

Materials and Methods

Animals

Specific pathogen-free, male Brown Norway rats, weighing 230-250 g, were purchased from Charles River Japan (Yokohama, Japan). All rats were housed at the facility in Biobubble Barrier Units under positive pressure.

Either rat interleukin-5 (IL-5, 0.2 ng/kg, R&D Systems, Minneapolis, MN) or PBS was injected intravenously one hour prior to administration of prostanoid receptor agonists. The agonists, including PGD_2, two CRTH2-specific agonists, DK-PGD_2 and 11-deoxy-11-methylene-15-keto-PGD_2 (MK-PGD_2), a DP-specific agonist, BW 245C, and a thromboxane A_2 receptor (TP)-specific agonist, I-BOP, were purchased from Cayman Chemical Co. (Ann Arbor, MI), and indomethacin was purchased from Nacalai Tesque Inc. (Kyoto, Japan). Each reagent was dissolved at a concentration of 0.01 - 1 mM in 100 μl PBS (pH 7.4) containing ethanol (PGD_2, BW 245C, I-BOP, indomethacin) or methylacetate (DK-PGD_2, MK-PGD_2). Since the eosinophil count in bronchoalveolar lavage fluid was equivalent regardless of the type of vehicle (PBS alone; $0.14 \pm 0.11 \times 10^4$ cells/ml, n = 3, PBS with ethanol; $0.27 \pm 0.11 \times 10^4$ cells/ml, n = 3, PBS with methylacetate; $0.47 \pm 0.05 \times 10^4$ cells/ml, n = 4), the combined data from all vehicle-treated rats were used in the analysis. Tracheostomy was performed under anesthetization with intraperitoneal ketamine (100 mg/kg) and xylazine (10 mg/kg), and the agonists or vehicles were sprayed intratracheally using a MicroSprayer™ Aerosolizer (model IA-1C with FMJ-250 high pressure syringe; Penn-Century, Philadelphia, PA). After the neck incision was sutured, the rats were allowed to awaken and were then returned to the cages.

In some experiments, a CRTH2/TP antagonist, ramatroban [BAY-u3405,

(+)-(3R)-3-(4-fluorobenzenesulfonamido)-1,2,3,4-tetra-hydrocarbazole-9-propionic acid, 3 - 10 mg/kg in 0.5% methyl cellulose, p.o.], a DP antagonist, BW A868C (1 mg/kg in normal saline containing ethanol, i.v., Cayman Chemical Co.), or a TP antagonist, SQ29,548 (2.5 mg/kg in normal saline containing ethanol, i.v., Cayman Chemical Co.) was administered two hours prior to administration of agonists. A previous study demonstrated that 1 - 10 mg/kg ramatroban administered orally in male rats resulted in the peak plasma concentration of 0.16 - 4.6 mg/L (385 – 11,000 nM) (Boberg et al., 1997), which is far above the concentration of ramatroban to block PGD_2-binding to rat CRTH2 *in vitro* (IC_{50}; 45 nM) (Shichijo et al., 2003).

The experimental protocol was reviewed and approved by the Laboratory Animal Care and Use Committee of Keio University School of Medicine.

Bronchoalveolar lavage (BAL)

The rats were sacrificed by injection of an overdose of pentobarbital at 2, 8, and 24 hours after the agonists were administrated. The trachea was cannulated and the lungs were lavaged three times with 8 ml PBS (pH 7.4).

Total cells in BAL fluid were counted using a hemocytometer, and the cell types were determined on Diff-Quik-stained cytospin slides prepared with Auto Smear CF12D (Sakura Fineteck, Tokyo, Japan). Four hundred cells in duplicate slides were counted in a blinded fashion.

Histological examination

After BAL was performed, the chest was opened, and the pulmonary circulation was thoroughly flushed with PBS using a peristaltic pump (flow rate 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. Lung tissues were paraffin-embedded, and the sliced sections were stained with Giemsa stain. A semi-quantitative scoring system was used to grade the degree of eosinophil accumulation. Each bronchus or vessel was graded from 0 (no eosinophils) to 4 (abundant eosinophilic infiltration) in a blinded manner. Five bronchi and vessels were evaluated in each side of the lungs, and the lung score (0 - 4) represents the mean value of the scores of both lungs. The lungs were examined independently by three investigators, and the scores were averaged.

Peripheral blood cell count

In an independent experiment, the rats received intravenous injection of IL-5 (0.2 ng/kg) or PBS one hour prior to intratracheal administration of PGD_2 (100 nmoles/animal) or vehicle. A peripheral blood sample was collected every hour after administration of IL-5 to determine the kinetics of blood eosinophils. The blood sample (10 μl), 50%(w/v) phloxine B solution (40 μl), and 0.05% methylene blue solution (50 μl) were mixed gently for 15 minutes, and the number of total leukocytes and eosinophils was determined.

Statistical analysis

All values are expressed as mean and SEM. Comparisons were performed by one-way ANOVA followed by Bonferroni/Dunn procedure as a post hoc test. A *p*-value less than 0.05 was considered statistically significant (STATVIEW 1992-98, SAS Institute Inc., NC).

Results

Intratracheal administration of PGD_2 (100 nmoles/animal) following IL-5 pretreatment (0.2 ng/kg, i.v.) induced a marked increase in the number of eosinophils in BAL fluid, whereas treatment with either PGD_2 alone or IL-5 alone showed no effect on BAL eosinophilia (Figure 1). In order to determine whether the eosinophilia in BAL fluid merely reflects the number of peripheral blood eosinophils, we examined the kinetics of eosinophils in the peripheral blood. Intravenous administration of IL-5 induced a 3-4 fold increase in the number of eosinophils in the peripheral blood within 2 hours ($p < 0.05$, Figure 2). PGD_2, however, did not demonstrate any additive or synergistic effects with IL-5 on blood eosinophilia (Figure 2), indicating that PGD_2 is essential for the local eosinophil trafficking from peripheral blood to the airway. The number of eosinophils in BAL fluid was dependent on the dose of PGD_2 (1 - 100 nmoles /animal, $p < 0.01$ - 0.05, Figure 3A). Eosinophilia in BAL fluid was observed within 2 hours after administration of PGD_2, and the number of eosinophils had decreased to the baseline at 8 and 24 hours (Figure 3B).

The biological activities of PGD_2 are mediated through multiple eicosanoid receptors on the cell surface, including CRTH2, DP, and TP (Hamid-Bloomfield et al., 1990; Boie et al., 1995, Hirai et al., 2001; Gervais et al., 2001; Monneret et al., 2001). We thus examined the specific receptor essential for PGD_2-induced eosinophil accumulation in the lungs. Two PGD_2-derivaives specific for CRTH2 (DK-PGD_2, MK-PGD_2), at a dose of 100 nmoles/animal, demonstrated an equivalent effect on eosinophil accumulation in BAL fluid to that of 100 nmoles/animal PGD_2 (Figure 4). Furthermore, indomethacin, which has a significantly different structure from PGD_2 or

its derivatives but exhibits similar agonistic activity for CRTH2 (Hirai et al., 2002), also showed potent eosinophil chemotactic activity *in vivo* (Figure 4). In contrast, a DP agonist, BW 245C (100 nmoles/animal), or a TP agonist, I-BOP (100 nmoles/animal), demonstrated little effect on eosinophil accumulation in BAL fluid (Figure 4). PGD_2-induced BAL eosinophilia was significantly inhibited by pretreatment with a CRTH2/TP antagonist, ramatroban, in a dose-dependent manner (3 – 10 mg/kg, $p < 0.05$ – 0.01, Figure 5A). Ramatroban (10 mg/kg) also inhibited the accumulation of eosinophils induced by CRTH2 agonists (DK-PGD_2, MK-PGD_2, indomethacin, $p < 0.01$, Figure 5B). In contrast, a TP-specific antagonist, SQ29,548, or a DP antagonist, BW A868C, did not inhibit the effects of PGD_2 on BAL eosinophilia (Figure 5A).

Histological examination demonstrated that intratracheal administration of PGD_2 in rats pretreated with IL-5 resulted in significant eosinophil accumulation in the perivascular and peribronchial spaces ($p < 0.01$, Figures 6, 7). Neither IL-5 nor PGD_2 alone could induce accumulation of eosinophils around bronchi or blood vessels. Treatment with ramatroban (10 mg/kg) almost completely abolished PGD_2-induced eosinophil accumulation in the lungs (data not shown).

Discussion

CRTH2, a newly-identified PGD_2 receptor, has been demonstrated to mediate PGD_2-induced mobilization and activation of inflammatory cells such as eosinophils *in vitro* (Gervais et al., 2001; Hirai et al., 2001; Monneret et al., 2001). Recent studies have demonstrated that an intravenous injection of a CRTH2 agonist such as Δ^{12}-PGJ_2 or DK-PGD_2 induced eosinophil recruitment from the bone marrow into the bloodstream (Heinemann et al., 2003; Shichijo et al., 2003). The present study clearly demonstrated that CRTH2 is also essential for PGD_2–induced recruitment of eosinophils from the bloodstream into organs.

Since PGD_2 can activate multiple receptors including CRTH2, DP, and TP (Hamid-Bloomfield et al., 1990; Boie et al., 1995, Hirai et al., 2001; Gervais et al., 2001; Monneret et al., 2001), we used a combination of receptor-specific agonists and antagonists to confirm the role of CRTH2. As CRTH2 agonists, we used DK-PGD_2, a well-established CRTH2-specific agonist *in vitro*, and MK-PGD_2, a stable analog of DK-PGD_2, although their specificity *in vivo* is not known. We thus performed additional experiments using another compound, indomethacin. Indomethacin has a significantly different structure from PGD_2 or its derivatives such as DK-PGD_2 and MK-PGD_2, but has been demonstrated to exhibit potent agonistic activity for CRTH2 (Hirai et al., 2002). All the CRTH2 agonists including indomethacin demonstrated equivalent activity to induce pulmonary eosinophilia to that of PGD_2, while a DP-specific agonist, BW 245C, or a TP-specific agonist, I-BOP, did not have such activity. Furthermore, a CRTH2/TP dual antagonist, ramatroban (3 – 10 mg/kg), abolished the effects of PGD_2 and all the CRTH2-specific agonists on BAL eosinophilia, but neither a DP antagonist, BW A868C, nor a TP antagonist, SQ29,548, did. We thus

concluded that PGD_2 in the airway lumen can attract eosinophils through the activation of CRTH2.

Previous studies demonstrated that an intravenous injection of PGD_2 or a CRTH2 agonist can induce peripheral blood eosinophilia (Heinemann et al., 2003; Shichijo et al., 2003). In the present study, however, intratracheally administered PGD_2, at a dose that induced significant BAL eosinophilia, exhibited no effect on the number of blood eosinophils. The discrepancy is possibly due to the difference in dose of agonists required to induce eosinophilia in peripheral blood and in local organs. Shichijo et al. reported that at least 100 μg DK-PGD_2 per rat (3×10^{-7} mol/rat) was necessary to induce peripheral blood eosinophilia when administered intravenously (Shichijo et al., 2003). In the present study, PGD_2 and CRTH2 agonists administered intratracheally induced significant eosinophilia in BAL at a lower dose of 10^{-9} - 10^{-7} mol/rat.

Intratracheally administered PGD_2 alone could not induce pulmonary eosinophilia as previously reported (Fujitani et al., 2002; Honda et al., 2003). We thus examined the synergistic activity of PGD_2 and systemic IL-5. Such an interaction between eosinophil chmoattractants and IL-5 in eosinophil trafficking to peripheral organs has been thoroughly examined for eotaxin, a well-established eosinophilic CC chemokine. Eotaxin-induced accumulation of eosinophils in the skin or lungs of guinea pigs or mice is amplified by either intravenous administration of IL-5 or overexpression of the IL-5 gene, but is completely abolished in IL-5-deficient mice (Collins et al., 1995; Rothenberg et al., 1996; Mould et al., 1997). Peripheral blood eosinophilia alone induced by adoptive transfer of eosinophils could not mimic the effects of systemic IL-5, suggesting that priming of circulating eosinophils by IL-5 is essential for eotaxin-induced tissue eosinophilia. In contrast, IL-5 has been shown to desensitize

eosinophils to PGD_2 by downregulating the expression of CRTH2 (Hamada et al., 2004). Similar phenomenon has been observed *in vivo*; eosinophils isolated from IL-5 transgenic mice can not bind to PGD_2 (Hirai et al., 2003). Further study is required to determine the interaction of PGD_2 and IL-5 during the induction of pulmonary eosinophilia.

Fukuyama and colleagues (Fukuyama et al., 2000) previously reported that intratracheally-administered human eotaxin induced airway eosinophilia in guinea pigs. The magnitude of airway eosinophilia induced by eotaxin (2 - 200 nmoles/animals) was almost equivalent to that induced by PGD_2 (100 nmoles/animals) in the present study, but more persistent. Increased number of eosinophils in BAL fluid was observed within 6 hours and persisted for 7 days in the eotaxin-injected guinea pigs, while PGD_2-induced airway eosinophilia disappeared within 8 hours in our model. However, sustained PGD_2 release during the early and late phase of allergic inflammation, which has been observed in the airways exposed to an allergen in animal models (Fujitani et al., 2002; Oguma et al., 2002) and in atopic subjects (O'sullivan et al., 1998; Bochenek et al., 2004), may result in prolonged airway eosinophilia. This is supported by the fact that a CRTH2/TP antagonist, ramatroban, effectively suppressed the airway eosinophil accumulation in sensitized guinea pigs even at 24 hours after the final allergen exposure (Nagai et al., 1995). There may be an alternative pathway for PGD_2 to induce eosinophil accumulation in the late phase of allergic inflammation, which was not examined in the present study. In a murine model of asthma, an inhalation of aerosolized PGD_2 induced the expression of macrophage-derived chemokine (MDC, CCL22) through an unidentified PGD_2 receptor on bronchial epithelial cells, and recruited Th2 lymphocytes into the airway (Honda et al., 2003). PGD_2 may then

enhance the production of eosinophilic cytokines such as IL-4, IL-5, and IL-13 from activated Th2 lymphocytes via CRTH2 receptor (Tanaka et al., 2004).

In conclusion, the present study demonstrated that CRTH2 receptor activation by PGD_2 released at the site of inflammation is essential for the pathogenesis of eosinophilic airway inflammation, suggesting that PGD_2 synthase inhibitors or CRTH2 antagonists can be useful to control allergic airway diseases such as asthma and allergic rhinitis.

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Reprint request to Koichiro Asano, M. D. Division of Pulmonary Medicine, Department of Medicine, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan

Figure Legends

Figure 1.

Total and differential leukocyte counts in BAL fluid.

BAL was performed at two hours after intratracheal administration of PGD_2 (100 nmoles/animal) or vehicle in IL-5 (0.2 ng/kg)- or PBS-pretreated animals. PBS/vehicle group (open bars, n = 3), IL-5/vehicle group (hatched bars, n = 10), PBS/PGD_2 group (dotted bars, n = 4), IL-5/PGD_2 group (closed bars, n = 11). Mean + SEM. **: $p < 0.01$, compared to PBS/vehicle group.

Figure 2.

Kinetics of eosinophils in peripheral blood.

Rats received intravenous injection of IL-5 or PBS one hour prior to intratracheal administration of PGD_2 (100 nmoles/animal) or vehicle (time 0). Peripheral blood samples were obtained every hour after administration of IL-5, and the number of eosinophils was counted. PBS/vehicle group (closed circles, n = 3), IL-5/vehicle group (open circles, n = 3), PBS/PGD_2 group (closed triangles, n = 3), IL-5/PGD_2 group (open triangles, n = 3). Mean + SEM. *: $p < 0.05$, compared to PBS/vehicle group.

Figure 3.

(A) PGD_2 dose-response of BAL eosinophilia two hours after intratracheal administration of PGD_2. PGD_2 (1 - 100 nmoles/animal) increased the number of eosinophils in BAL fluid from IL-5-pretreated rats in a dose-dependent manner. n = 3 - 11. *: $p < 0.05$, **: $p < 0.01$, compared to vehicle-injected animals.

(B) Time course of BAL eosinophilia following intravenous IL-5/intratracheal PGD_2

(100 nmoles/animal) treatment. PBS/vehicle group (open bars, n = 3 - 7), IL-5/PGD_2 group (hatched bars, n = 3 - 11). Mean + SEM. **: $p < 0.01$, compared to PBS/vehicle group.

Figure 4.

Effects of various prostanoid receptor agonists on BAL eosinophilia. BAL was performed two hours after PGD_2 (n = 11), CRTH2 agonist (DK-PGD_2, MK-PGD_2, indomethacin, n = 6 each), DP agonist (BW 245C, n = 6), TP agonist (I-BOP, n = 3) or vehicle (n = 10) was administered intratracheally in IL-5-pretreated animals. All the agonists were administered at a dose of 100 nmoles/animal. Mean + SEM. **: $p < 0.01$, compared to vehicle group.

Figure 5.

(A) Effects of various prostanoid receptor antagonists on BAL eosinophilia in IL-5/PGD_2-treated rats. Either CRTH2/TP antagonist (ramatroban, 3 - 10 mg/kg p.o., n = 3 each), TP antagonist (SQ29,548, 2.5 mg/kg i.v., n = 3), DP antagonist (BW A868C, 1 mg/kg i.v., n = 5), or vehicle (n = 4) was administered two hours prior to intratracheal injection of PGD_2. PBS-based vehicle, instead of PGD_2, was administered intratracheally in the PBS group (n = 10). Mean + SEM. *: $p < 0.05$, **: $p < 0.01$, compared to PBS group. ††: $p < 0.01$, compared to vehicle group.

(B) Effects of ramatroban on CRTH2 agonist-induced BAL eosinophilia. Either ramatroban (hatched columns, 10 mg/kg p.o., n = 3 - 7) or vehicle (closed columns, n = 6 – 11) was administered one hour prior to intratracheal injection of CRTH2 agonist (100 nmoles/animal). Mean + SEM. **: $p < 0.01$, compared to vehicle group.

Figure 6.

Histological examination of lungs from rats treated with intravenous IL-5 (0.2 ng/kg), intratracheal PGD_2 (100 nmoles/animal), or both. Giemsa stain. PBS/vehicle (A), PBS/PGD_2 (B), IL-5/vehicle (C), IL-5/PGD_2 (D). Original magnification, x100. V: vessel, B: bronchus.

Figure 7.

Semi-quantitative histological scoring of eosinophil accumulation in peribronchial (A) and perivascular (B) spaces. The degree of eosinophil accumulation in each bronchus or vessel was graded from 0 (no eosinophils) to 4 (marked eosinophil infiltration). Five bronchi and vessels were evaluated in each side of lungs, and the total lung score (0 - 40) represents the sum of the scores of both lungs. Bars represent mean values. n = 4 each. **: $p < 0.01$, compared to PBS/vehicle group.

Figure 1

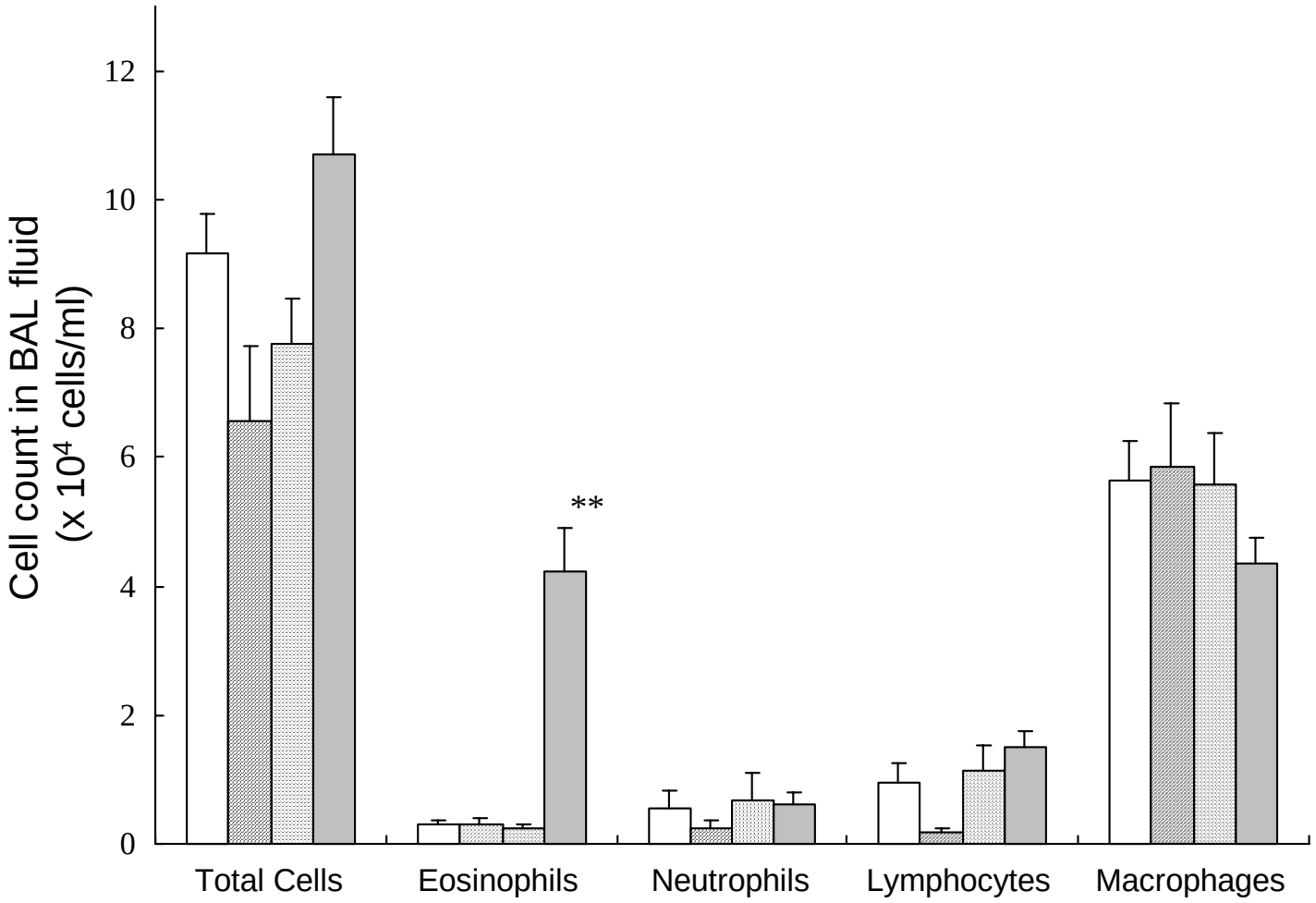

Figure 2

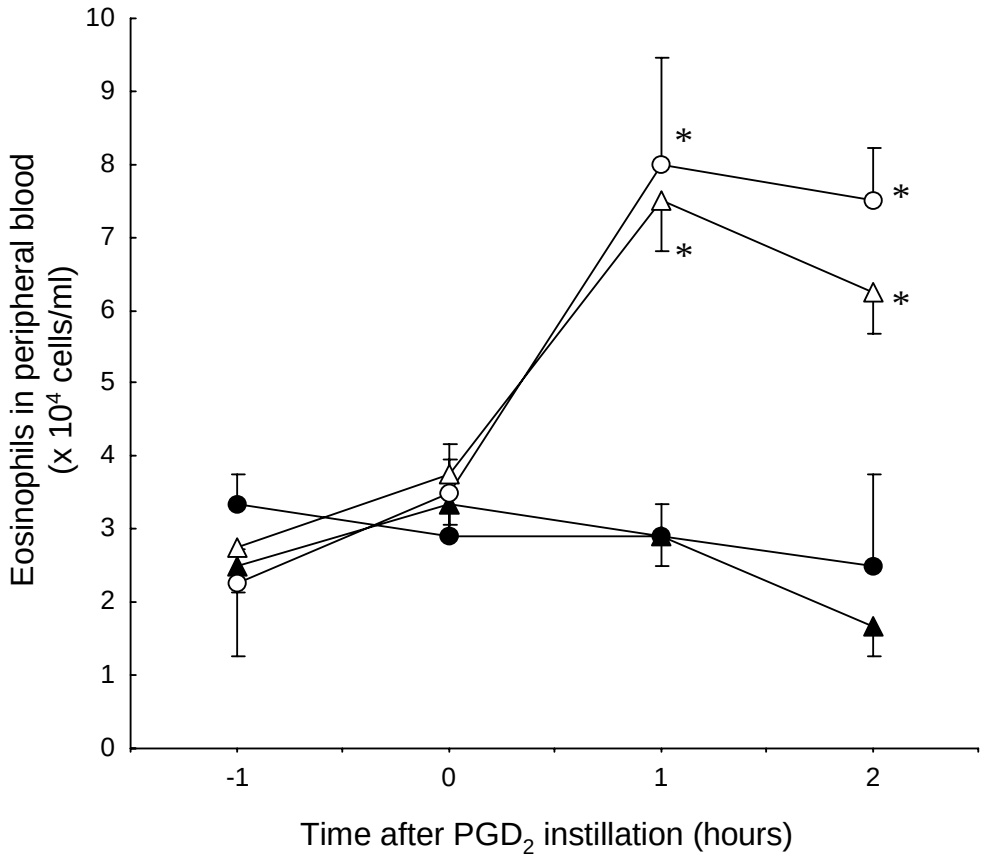

Figure 3

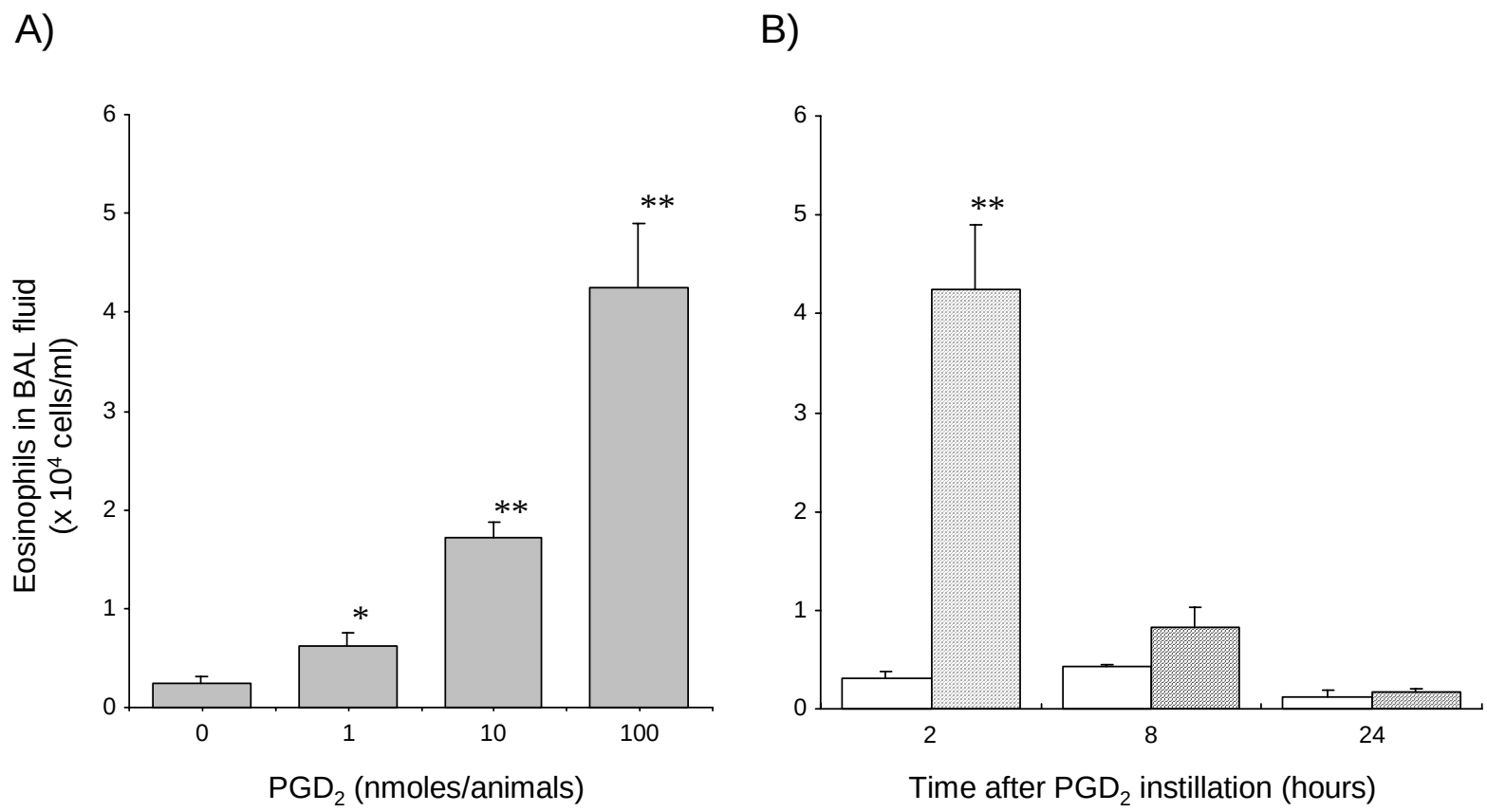

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Figure 4

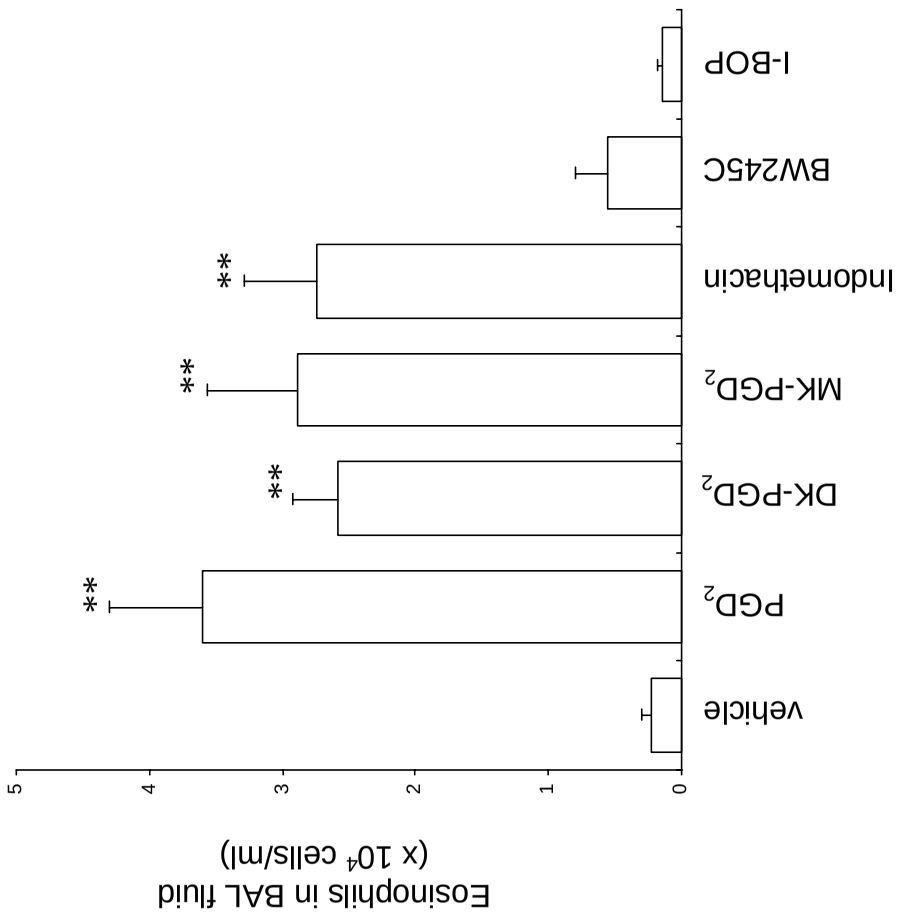
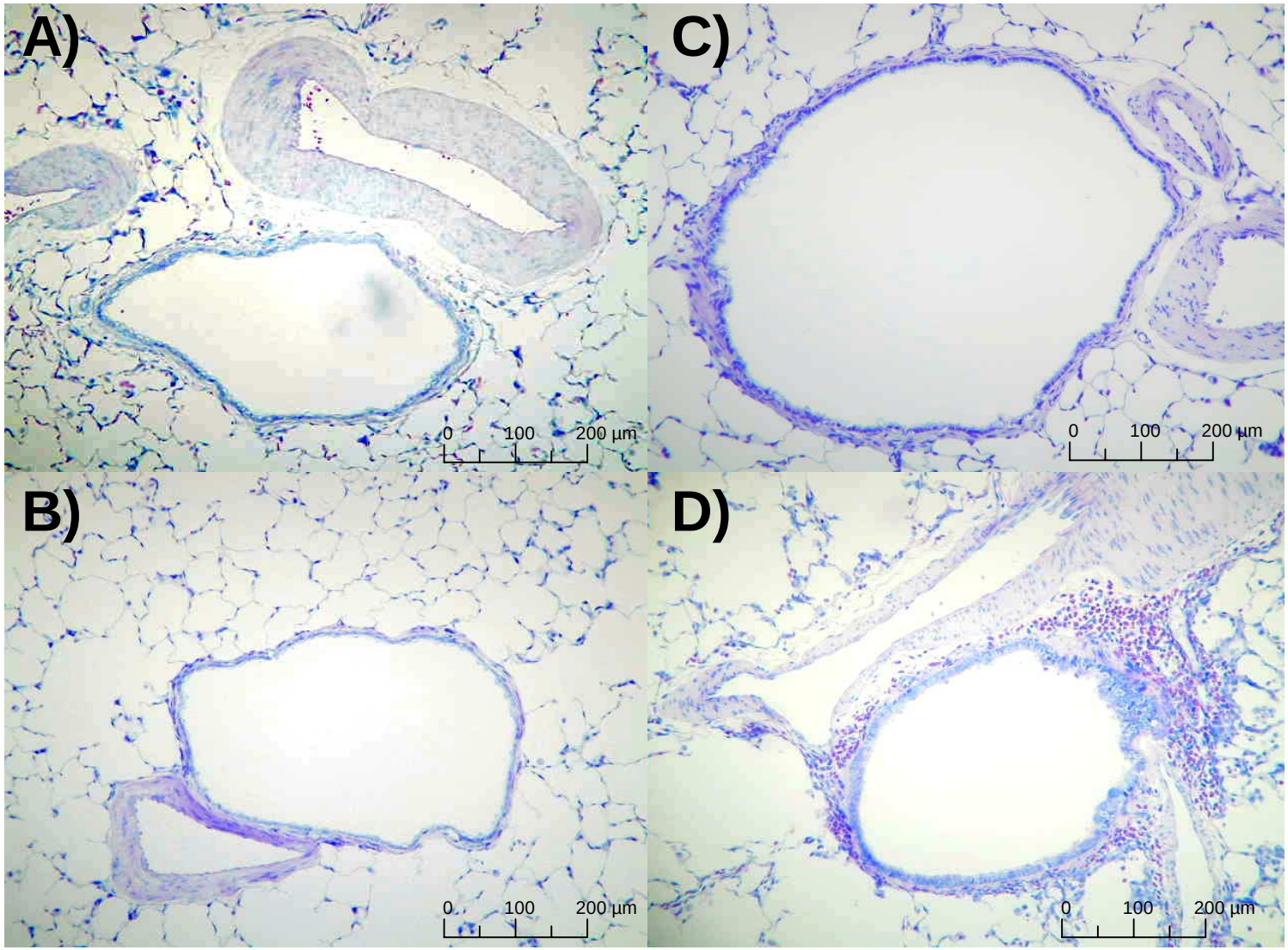


Figure 5

Figure 6



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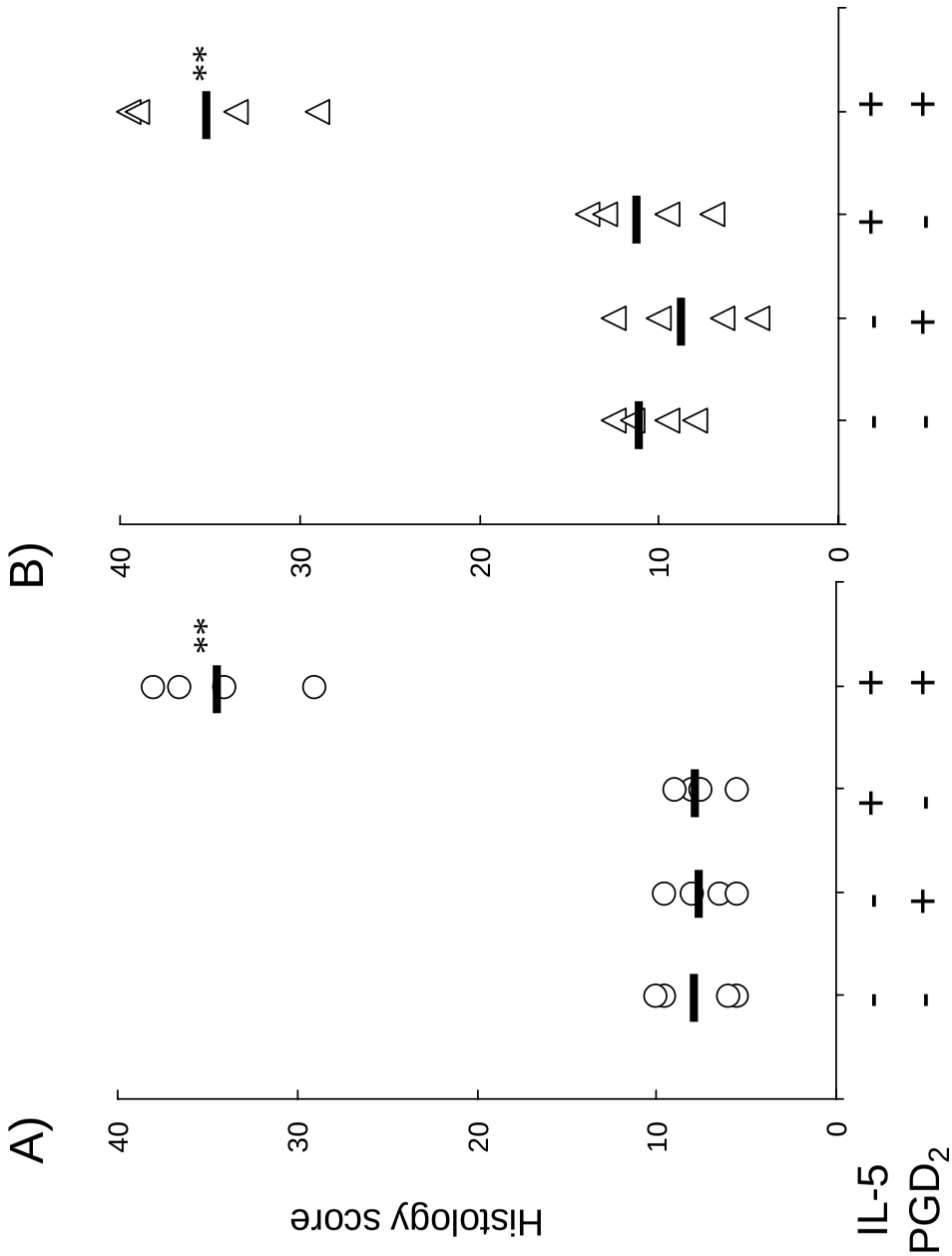

Figure 7