ORIGINAL ARTICLE

Prostaglandin I_2 Signaling and Inhibition of Group 2 Innate Lymphoid Cell Responses

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Abstract

Rationale: Group 2 innate lymphoid cells (ILC2s) robustly produce IL-5 and IL-13, cytokines central to the asthma phenotype; however, the effect of prostaglandin (PG) I_2 on ILC2 function is unknown.

Objectives: To determine the effect of PGI_2 on mouse and human ILC2 cytokine expression *in vitro* and the effect of endogenous PGI_2 and the PGI_2 analog cicaprost on lung ILC2s *in vivo*.

Methods: Flow-sorted bone marrow ILC2s of wild-type (WT) and PGI_2 receptor–deficient ($IP^{-/-}$) mice were cultured with IL-33 and treated with the PGI_2 analog cicaprost. WT and $IP^{-/-}$ mice were challenged intranasally with *Alternaria alternata* extract for 4 consecutive days to induce ILC2 responses, and these were quantified. Prior to *A. alternata* extract, challenged WT mice were treated with cicaprost. Human flow-sorted peripheral blood ILC2s were cultured with IL-33 and IL-2 and treated with the PGI_2 analog cicaprost.

Measurement and Main Results: We demonstrate that PGI_2 inhibits IL-5 and IL-13 protein expression by IL-33–stimulated ILC2s purified from mouse bone marrow in a manner that was dependent on signaling through the PGI_2 receptor IP. In a mouse model of 4 consecutive days of airway challenge with an extract of *A. alternata*, a fungal aeroallergen associated with severe asthma exacerbations, endogenous PGI_2 signaling significantly inhibited lung IL-5 and IL-13 protein expression, and reduced the number of lung IL-5– and IL-13–expressing ILC2s, as well as the mean fluorescence intensity of IL-5 and IL-13 staining. In addition, exogenous administration of a PGI_2 analog inhibited *Alternaria* extract–induced lung IL-5 and IL-13 protein expression, and reduced the number of lung IL-5– and IL-13–expressing ILC2s and the mean fluorescence intensity of IL-5 and IL-13 staining. Finally, a PGI_2 analog inhibited IL-5 and IL-13 expression by human ILC2s that were stimulated with IL-2 and IL-33.

Conclusions: These results suggest that PGI_2 may be a potential therapy to reduce the ILC2 response to protease-containing aeroallergens, such as *Alternaria*.

Keywords: ILC2; PGI_2; asthma; allergy

Over the last four decades, the prevalence of allergic rhinitis, asthma, and atopic eczema increased markedly in developed countries (1, 2). Mounting evidence suggests that drugs inhibiting cyclooxygenase (COX) enzymes in the arachidonic acid metabolic pathway may contribute to the increased allergy prevalence (3–5). Animal studies confirmed that COX inhibition increased allergic sensitization; augmented allergic airway inflammation; and enhanced lung expression of IL-4, IL-5, and IL-13

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At a Glance Commentary

Scientific Knowledge on the Subject: Group 2 innate lymphoid cells (ILC2s) secrete large amounts of cytokines that are important in the development and maintenance of allergic inflammation.

What This Study Adds to the Field: We have discovered that prostaglandin I_2, inhibited the function of mouse and human ILC2s. Our findings suggest that prostaglandin I_2 may be used therapeutically to reduce allergic inflammation by inhibiting ILC2 cytokine secretion and proliferation.

cytokines expressed by $CD4^+$ Th2 cells, supporting a role for the COX pathway in regulating allergic inflammation (6–13). Because COX inhibition increases allergic inflammation in humans and experimental animal models, this suggests that a COX product restrains allergen-induced inflammatory responses and may be a novel treatment strategy for allergic diseases.

Indeed, studies performed by our laboratory and other investigators revealed that the COX product prostaglandin (PG) I_2, which signals through the G protein–coupled receptor IP, was a critical inhibitor of the adaptive immune response in animal models of allergic lung inflammation (14–20). IP predominantly couples to a G_s-type G protein, leading to an increase in cAMP (21). In addition, IP is capable of coupling to G_q-dependent phosphoinositide turnover and G_i-dependent inhibition of cAMP (22, 23). Thus, mouse IP is capable of coupling to G_s, G_q, and G_i, with the latter two dependent on agonist-induced, G_s-dependent phosphorylation by protein kinase A (PKA) (24). To date, no studies have investigated the effect of PGI_2 signaling on the innate allergic inflammatory response that is mediated by the recently described group 2 innate lymphoid cells (ILC2s), which are resident in the lung, skin, gut, and other organs (25).

ILC2s are lineage negative (lin^-) in that they do not express T-cell, B-cell, macrophage, or dendritic cell (DC) markers (26), yet these cells have surface expression of CD25 (IL-2Rα) and CD127 (IL-7Rα) (26–30). ILC2s produce Th2-type cytokines, such as IL-5, at a 10-fold greater level than $CD4^+$ Th2 cells, yet low amounts of IL-4 and IFN-γ (31). ILC2 expression of common γ chain of the IL-2 receptor and the signal transduction factor Id2 are critical for ILC2 development (25). Although the exact contribution of ILC2s in the global allergic response is unknown, this potent Th2-type cytokine production on a per cell basis suggests that these cells are important initiators or amplifiers of allergen-induced inflammation. *In vivo* experiments revealed that lung ILC2s have a critical role in rapid Th2-type inflammation in response to protease aeroallergens, such as *Alternaria alternata*, which is associated with severe exacerbations of asthma (26, 32–35). Although IL-33, IL-25, and thymic stromal lymphopoietin are all expressed by epithelial cells in response to proteases, recent studies reveal that IL-33 may be the most important cytokine that drives Th2-type cytokine expression by lung ILC2s (26, 32).

Very few pharmacologic agents and no PGs are known to inhibit cytokine production by ILC2s. The inhaled PGI_2 analog, iloprost, has been well tolerated and very effective for pulmonary hypertension, and its use could potentially be extended for the treatment of allergic airway diseases, such as asthma (36). Therefore, we tested the hypotheses that PGI_2 is an important negative regulator of human and mouse ILC2s *in vitro*, and that endogenous and exogenous PGI_2 inhibit ILC2 function in a novel acute *Alternaria*-challenge mouse model that elicits a robust Th2-type cytokine production in the lung before the appearance of adaptive immune cells.

Methods

Mice

Wild-type (WT) BALB/c mice were obtained from Charles Rivers (Wilmington, MA). $IP^{-/-}$ mice were backcrossed to a BALB/c background for 10 generations (21). Age-matched WT BALB/c mice and $IP^{-/-}$ mice were used at 8–12 weeks old. Animal experiments were reviewed; approved by the Institutional Animal Care and Use Committee at Vanderbilt University; and were conducted according to the guidelines for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources, National Research Council.

Isolation of Mouse ILC2s

lin^- bone marrow cells were isolated using a magnetic cell separation method (Miltenyi Corp., Cambridge, MA). ILC2s were further purified by flow cytometry after the lin^- cells were stained with fluorochrome-labeled anti-CD3, anti-CD25, and anti-CD127 antibodies. The purified $lin^-CD3^-CD25^+CD127^+$ cells were cultured in round bottom 96-well plates (5,000 cells/well) and treated with IL-33 (10 ng/ml) or phosphate-buffered saline (PBS). ILC2s were also treated with IL-33 in the presence of either vehicle or the PGI_2 analog cicaprost (10 nM or 100 nM) for 3 days. Cicaprost was kindly and generously provided by Dr. Manuela Huebner (Bayer HealthCare, Berlin, Germany). Additional detail on the method of isolation of ILC2s is provided in the online supplement.

IP Expression on Lung ILC2s

Whole lung cells were harvested from WT and $IP^{-/-}$ mice. The cells were enriched using the Miltenyi lineage negative isolation kit and stained with fluorochrome-labeled antibodies for CD3, CD45, CD25, and CD127. A mouse IP receptor-specific polyclonal antibody (Cayman Chemical, Ann Arbor, MI) and PE-conjugated secondary antibody (Biolegend, San Diego, CA) were also used for cell staining to detect IP protein expression on ILC2s by flow cytometry. ILC2s were gated as lin^- $CD3^-CD45^+CD25^+CD127^+$ cells before IP protein detection by flow cytometry.

ILC2 Proliferation

WT and $IP^{-/-}$ ILC2s ($lin^-CD25^+CD127^+$ cells) were cultured with IL-33 (10 ng/ml) or PBS in round bottom 96-well plates (5,000 cells/well) and treated with cicaprost (10 nM) or vehicle for 3 days. Live cells were counted by trypan blue staining after 3 days. In some experiments, the cells were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE) before culture. CFSE intensity was determined by flow cytometry for cell proliferation analyses.

Droplet Digital Polymerase Chain Reaction

Sorted bone marrow ILC2s were cultured for 3 days, counted, and harvested. Cell Amp whole transcriptome amplification kit (TAKARA Bio Inc., Shiga, Japan) was used for direct cell lysis and cDNA generation. Sample preparation for droplet generation was performed using the Droplet Digital

polymerase chain reaction (PCR) supermix for probes (Bio-Rad, Hercules, CA). In a 20-μl PCR reaction, 1 μl of cDNA was added to 10 μl of 2× supermix with the addition of mouse Id2 and glyceraldehyde phosphate dehydrogenase (GAPDH) primers at final concentrations of 900 and 250 nM, respectively. The reaction mixture was converted into droplets using the QX200 droplet generator (Bio-Rad). The resultant droplets were then subjected to amplification as follows: one cycle at 95°C for 10 minutes, 40 cycles at 94°C for 30 seconds, and 60°C for 1 minute, and a final cycle at 98°C for 10 minutes with a ramp speed of 2.5°C/s in a C1000 Touch Thermal Cycler (Bio-Rad). After PCR, the reactions were loaded onto the QX200 Droplet Digital reader, and analysis was performed using QuantaSoft software (Bio-Rad). Mouse Id2 (Mm00711781_m1), mouse GAPDH (Mm99999915_g1), and human Id2 (hs04187239_m1) primers were purchased from Applied Biosystems (Foster City, CA).

Rp-8-Br-cAMPS Treatment

8-Bromoadenosine-3′,5′-cyclic monophosphorothioate, Rp-isomer (Rp-8-Br-cAMPS) was purchased from Sigma-Aldrich (St. Louis, MO). ILC2s were pretreated with Rp-8-Br-cAMPS (0.1, 1, and 10 μM) for 1 hour at 37°C before incubation with cicaprost (0.5 nM) as previously described (20).

In Vivo Alternaria Extract Challenge

WT and $IP^{-/-}$ mice were anesthetized with ketamine/xylazine and then challenged intranasally with either intranasal 100 μl PBS or *Alternaria* extract (5 μg) in 100 μl PBS daily for 4 consecutive days. Twenty-four hours after the last challenge, the mice were killed. In some experiments, whole lungs were digested and both cell surface marker staining and intracellular cytokine staining was performed to enumerate the IL-5– and IL-13–expressing ILC2s. In other experiments, WT mice were anesthetized with ketamine/xylazine and treated with either 50 μl PBS or cicaprost (2.5 μg) in 50 μl PBS 15 minutes before each challenge with either intranasal 50 μl PBS or *Alternaria* (5 μg) in 50 μl PBS daily for 4 consecutive days. Mucus was assessed by histopathology with periodic acid Schiff (PAS) staining 48 hours after the last *Alternaria* extract challenge.

Histologic Analyses of Lung Sections

Histopathology was performed as previously described (10).

Cytokine Measurements

Quantikine ELISA kits from R&D Inc. (Minneapolis, MN) were used to measure the protein levels of cytokines (IL-5 and IL-13) according to the manufacturer's instructions.

Isolation of Human ILC2s

White blood cells were flushed from six RC2D blood filters obtained from the Nashville Area Chapter of the American Red Cross. lin^- cells were first isolated using a lineage cell depletion kit and a Miltenyi microbead isolation system. lin^- cells were stained with propidium iodide and fluorochrome-labeled antibodies against CD3, CD25, and CD127, and live $CD3^-CD25^+CD127^+$ cells were sorted by flow cytometry. Purified ILC2s were cultured for 6 days in IL-33 (10 ng/ml) and IL-2 (10 ng/ml) with either cicaprost (10 or 100 nM) or vehicle control (water). Cell culture supernatants were examined for IL-5 and IL-13 protein expression by ELISA.

Statistical Analysis

The *P* values were calculated by using unpaired Student *t* test or one-way analysis of variance with Bonferroni *post hoc* test. Error bars represent SEM. Values of *P* less than 0.05 were considered significant.

Results

IL-5 and IL-13 Expression by Mouse ILC2s Was Inhibited by the PGI_2 Analog Cicaprost in an IP-Specific Manner

We hypothesized that mouse ILC2s express IP and that cicaprost inhibits IL-5 and IL-13 protein expression by mouse ILC2s. To test this hypothesis, we isolated mouse ILC2s from bone marrow. lin^- cells were isolated from mouse bone marrow using a commercially available kit (Miltenyi Corp.). The cells were stained with propidium iodide and viable $lin^-CD3^-CD25^+CD127^+$ cells were cell sorted by flow cytometry. The gating strategy for isolation of ILC2s from bone marrow cells is shown in Figure E1 in the online supplement. Freshly prepared lung ILC2s from WT mice expressed IP as determined by flow cytometry (Figure 1A). In culture, IL-33 increased bone marrow ILC2 protein expression of IL-5 (Figure 1B) and IL-13 (Figure 1C), and IL-33–stimulated secretion of both of these cytokines was statistically significantly inhibited by cicaprost at both 10 and 100 nM. The effect of cicaprost inhibition on both IL-5 and IL-13 was IP specific because cicaprost had no effect on either IL-5 or IL-13 protein expression by ILC2s from $IP^{-/-}$ mice. The time course for this experiment in shown in Figure 1D.

Cicaprost Inhibited IL-33–induced ILC2 Proliferation

Because cicaprost significantly inhibited IL-33–induced IL-5 and IL-13 production, we assessed cicaprost regulation of Id2 mRNA expression by mouse ILC2s by Digital Droplet PCR. As shown in Figure E2A, cicaprost significantly inhibited IL-33–induced ILC2 expression of Id2, even at very low concentrations of cicaprost. Because cicaprost inhibited IL-33 induction of both ILC2 cytokine production and Id2 expression, we determined the effect of cicaprost on cell viability. As shown in Figure E2B, cicaprost 10 nM significantly reduced the number of live ILC2s as measured by trypan blue staining in an IP-specific fashion, because cicaprost had no effect on the cell viability of ILC2s from $IP^{-/-}$ mice.

We confirmed this finding by performing a proliferation assay in which mouse ILC2s were stained with CFSE and then cultured with IL-33 either in the absence or presence of cicaprost 10 nM. As shown in Figure E2C, ILC2s from WT mice had extensive proliferation when stimulated with IL-33; however, this was markedly reduced with cicaprost treatment. In contrast, cicaprost had no effect on IL-33–induced proliferation of ILC2s from $IP^{-/-}$ mice. Because cicaprost reduced cell viability in an IP-specific fashion, we sought to determine if cicaprost induced apoptosis. To answer this question, we examined markers of ILC2 apoptosis in culture with IL-33, either in the absence or presence of cicaprost 10 nM. We found that starting on Day 2 of culture, cicaprost induced cell death in WT ILC2s, but not $IP^{-/-}$ ILC2s, as defined by annexin V^+DAPI^+ staining (*see* Figure E2D). Surprisingly, we did not find that cicaprost 10 nM increased the number of annexin V^+DAPI^- cells, a phenotype that would indicate apoptosis. The mechanism of cicaprost-mediated, IP-specific cell death is one that we will continue to investigate in future studies.

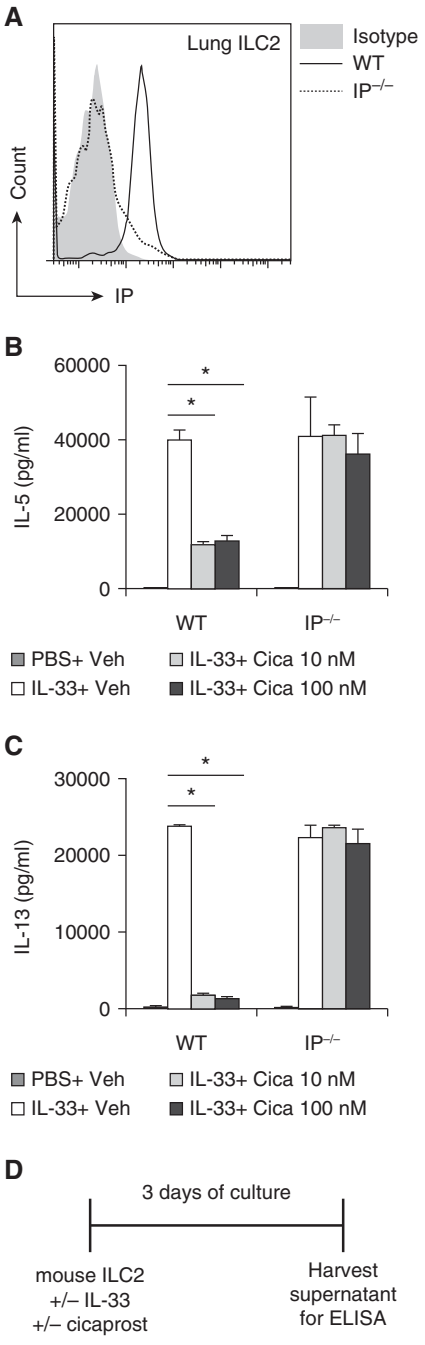

Figure 1. (*A*) Freshly prepared lung group 2 innate lymphoid cells (ILC2s) from wild-type (WT) mice expressed prostaglandin I_2 receptor (IP) as determined by flow cytometry. (*B*) The effect of IL-33 (10 ng/ml) and cicaprost (10 and 100 nM) on IL-5 protein expression by bone marrow ILC2s from WT and $IP^{-/-}$ mice (n = 4–5). (*C*) The effect of IL-33 (10 ng/ml) and cicaprost (10 and 100 nM) on IL-13 protein expression by bone marrow ILC2s from WT and $IP^{-/-}$ mice (n = 4–5). (*D*) Time course of the experiment. Cica = cicaprost; PBS = phosphate-buffered saline; Veh = vehicle. *$P < 0.05$.

Cicaprost Inhibited ILC2 Cytokine Production in a Partially PKA-Dependent Fashion

To determine the signaling mechanism by which cicaprost inhibited ILC2 cytokine production, we used Rp-8-Br-cAMP, a lipophilic analog of Rp-cAMPS that is a competitive inhibitor of PKA. As shown in Figure E2E, we found that RP-8-Br-cAMP 1 μM significantly increased IL-33–induced ILC2 production of IL-13 that had been inhibited by cicaprost 0.5 nM, but not to the level of IL-33–induced ILC2 production of IL-13 in the absence of cicaprost. Therefore, we conclude that effect of cicaprost in inhibiting IL-33–induced ILC2 production of IL-13 was partially, but not completely, dependent on the PKA pathway.

IP Deficiency Increased the Number of Mouse Lung ILC2s in Response to Airway *Alternaria alternata* Extract Challenge

Based on our finding that a PGI_2 analog inhibited IL-5 and IL-13 protein expression from IL-33–stimulated ILC2s in an IP-dependent manner, we hypothesized that endogenous PGI_2 reduces IL-5 and IL-13 production by lung ILC2s *in vivo*. To test this hypothesis, we challenged mice with either PBS or an extract of *A. alternata* antigen dissolved in PBS administered intranasally for 4 consecutive days and then killed the mice 24 hours later (protocol shown in Figure E3). Four days allows for an innate immune response, including ILC2s, but not an adaptive immune response. We chose an extract of *A. alternata* because it contains a cysteine protease that potently induces airway epithelial cells to produce IL-33 via its protease activity (34). This extract does not contain live *Alternaria*, but instead contains the epitopes that lead to IgE-mediated positive skin tests in persons sensitized to *Alternaria* and result in airway responsiveness in persons whose asthma is caused by *Alternaria* (37). We chose this time course with the consideration that there was unlikely to be a robust adaptive immune response after only 4 consecutive days of antigen challenge, and that this protocol would allow us to assess the innate immune response of which ILC2s are a component.

First we measured IL-5 and IL-13 in the bronchoalveolar lavage (BAL) fluid from WT and $IP^{-/-}$ mice. We found a statistically significant 2.5-fold increase in IL-5 and IL-13 protein expression in the BAL fluid from $IP^{-/-}$ mice (Figure 2A). We then enumerated the number of lung ILC2s following either 4 consecutive days of PBS or *Alternaria* extract challenge. The flow cytometry gating strategy is shown in Figure E4. There was a statistically significant increase in the percentage of lung ILC2s that expressed either IL-5 or IL-13 in both WT and $IP^{-/-}$ mice following *Alternaria* extract–challenged mice compared with those challenged with PBS (Figure 2C). There was a statistically significant twofold increase in the percentage of IL-5– and IL-13–expressing ILC2s and a significant ninefold increase in the number of IL-5– and IL-13–expressing ILC2s in the *Alternaria* extract–challenged $IP^{-/-}$ mice compared with the WT mice, although there was no difference in the number of IL-5– and IL-13–expressing ILC2s in the PBS-challenged $IP^{-/-}$ mice compared with the WT mice. In addition, there was a statistically significant increase in the mean fluorescence intensity (MFI) of both IL-5 and IL-13 staining in the lung ILC2s that expressed these cytokines in the *Alternaria*-challenged $IP^{-/-}$ mice compared with WT mice. The total number of ILC2s in the *Alternaria*-challenged $IP^{-/-}$ mice was significantly greater than *Alternaria*-challenged WT mice (*see* Figure E5A). ILC2s as a percentage of all $lin^{-}CD45^{+}$ cells are shown in Figure E5B.

To determine the contribution of $CD4^{+}$ cells to the IL-5 and IL-13 present in the total lung protein levels, flow cytometry with intracellular cytokine staining was performed (Figure 2D). Neither the percentage nor the number of $CD4^{+}IL\text{-}5^{+}$ and $CD4^{+}IL\text{-}13^{+}$ cells was statistically significantly different between the PBS-challenged WT mice and the PBS-challenged $IP^{-/-}$ mice, whereas the percentage and the number of $CD4^{+}IL\text{-}5^{+}$ and $CD4^{+}IL\text{-}13^{+}$ cells was statistically significantly increased in both the *Alternaria* extract–challenged WT and $IP^{-/-}$ mice compared with either of the PBS-challenged groups. However, there was no difference in either the percentage or the number of $CD4^{+}IL\text{-}5^{+}$ and $CD4^{+}IL\text{-}13^{+}$ cells between the *Alternaria* extract–challenged WT and $IP^{-/-}$ mice (Figure 2E). The MFI of IL-5 and IL-13 staining of the $CD4^{+}IL\text{-}5^{+}$ cells or $CD4^{+}IL\text{-}13^{+}$ cells, respectively, was not different between either PBS- or *Alternaria* extract–challenge for either the WT or $IP^{-/-}$

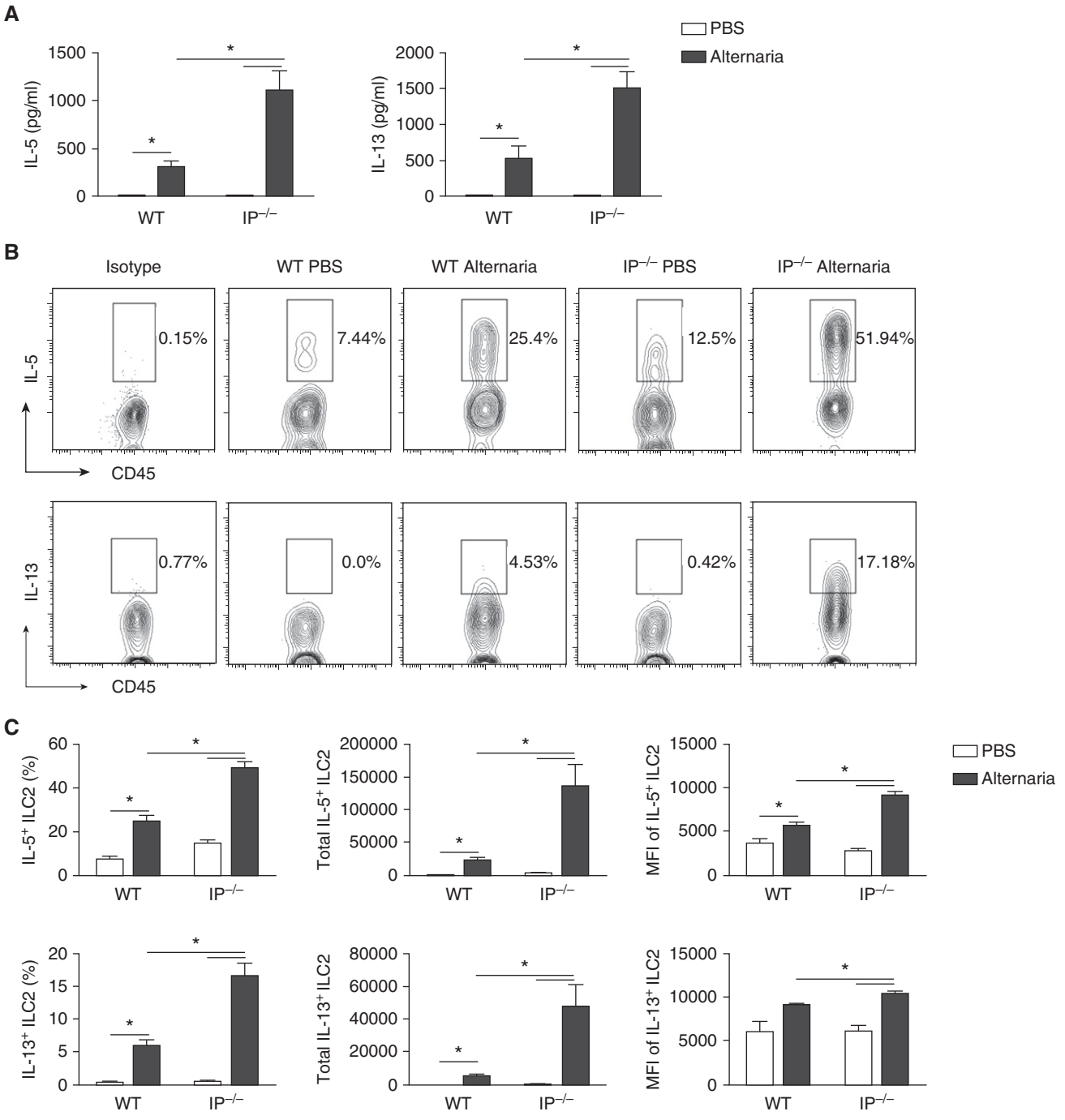

Figure 2. (*A*) IL-5 (*left*) and IL-13 (*right*) protein expression in bronchoalveolar lavage fluid from phosphate-buffered saline (PBS)- (n = 3) or *Alternaria* extract– (n = 5) challenged wild-type (WT) and prostaglandin I_2 receptor–deficient ($IP^{-/-}$) mice. (*B*) Representative plots of IL-5– and IL-13–expressing $lin^-CD25^+CD127^+CD45^+$ group 2 innate lymphoid cells (ILC2s) from PBS- or *Alternaria* extract–challenged WT and $IP^{-/-}$ mice. (*C*) The percentage, number, and MFI of $lin^-CD25^+CD127^+CD45^+IL\text{-}5^+$ and $lin^-CD25^+CD127^+CD45^+$ $IL\text{-}13^+$ ILC2s in PBS- (n = 4) or *Alternaria* extract– (n = 5) challenged WT and $IP^{-/-}$ mice. (*D*) Representative plots of IL-5– and IL-13–expressing $CD3^+CD4^+$ cells from PBS- or *Alternaria* extract–challenged WT and $IP^{-/-}$ mice. (*E*) The percentage, number, and IL-5 and IL-13 MFI of $CD3^+CD4^+$ cells in PBS- (n = 3) or *Alternaria* extract– (n = 6) challenged WT and $IP^{-/-}$ mice. *$P < 0.05$. MFI = mean fluorescence intensity.

mice. In the *Alternaria* extract–challenged $IP^{-/-}$ mice, there was a 50-fold increase in the IL-5–expressing ILC2s (Figure 2C) compared with IL- 5–expressing $CD4^+$ cells (Figure 2E), and a fivefold increase in the IL-13–expressing ILC2s (Figure 2C) compared with IL-13–expressing $CD4^+$ cells (Figure 2E).

These results reveal that endogenous PGI_2 signaling through IP significantly inhibits the number of lung ILC2s in response to *Alternaria* extract challenge and also negatively regulates IL-5 and IL-13 expression by lung ILC2s on a per cell basis in response to this same challenge. The

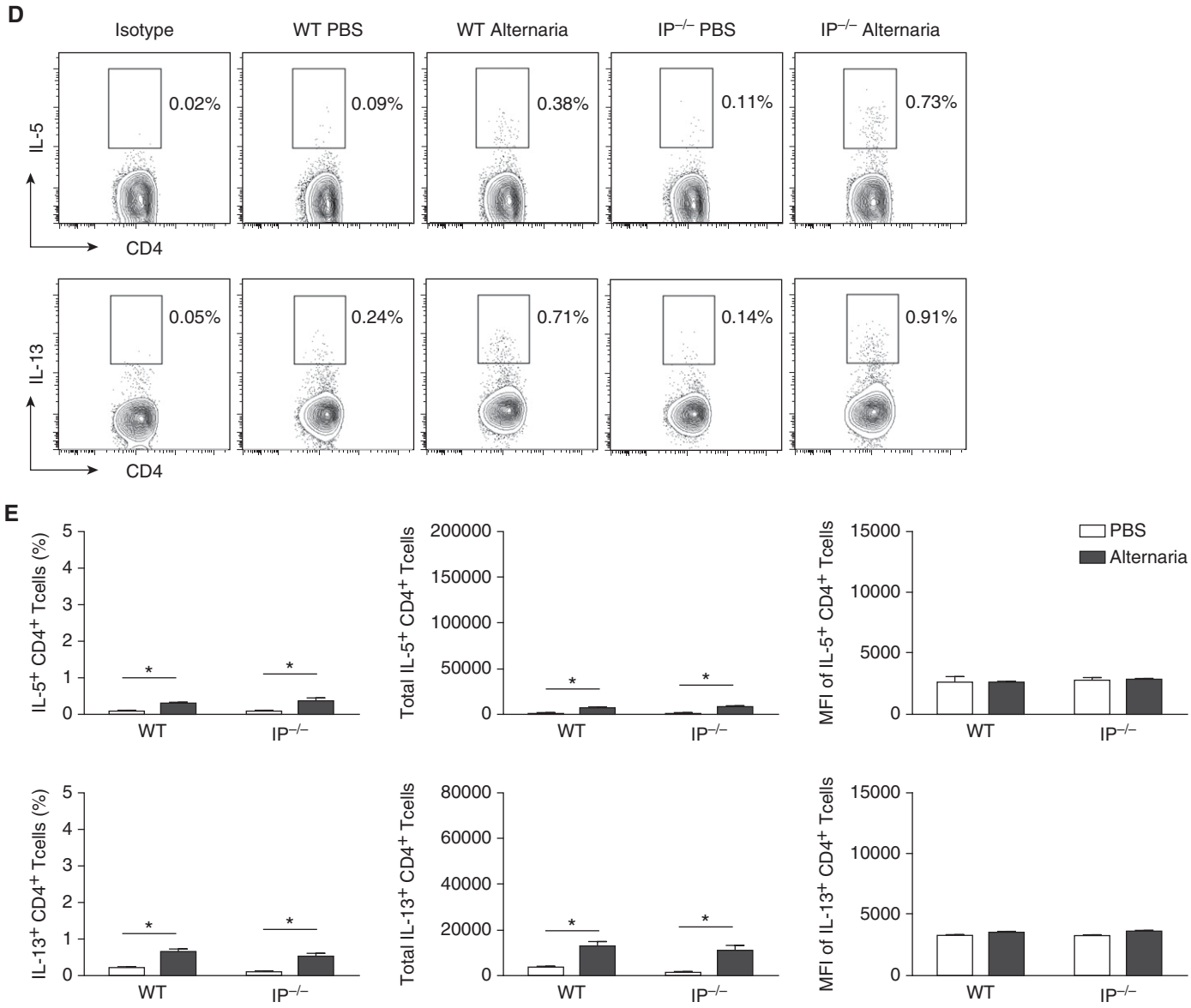

Figure 2. *(continued)*

results also support that ILC2s, but not $CD4^+$ cells, are the major producers of IL-5 and IL-13 in response to this 4-consecutive-day airway *Alternaria* extract challenge protocol. The concept that innate, and not adaptive, immunity is primarily operational in the allergic airway inflammation induced by the 4-consecutive-day allergen exposure model is further supported by the total serum IgE and antigen- specific IgG1 antibody measurements shown in Figure E6. In this experiment, WT and $IP^{-/-}$ mice challenged with *Alternaria* extract in the 4-consecutive-day challenge protocol had total serum IgE and antigen-specific IgG1 levels that were no different from mice challenged with PBS, signifying no quantifiable humoral immune response to antigen in this protocol. As a positive control for allergen-induced antibody production, WT mice were challenged with an *Alternaria* adaptive immunity protocol in which mice were challenged with *Alternaria* extract four times with each challenge occurring on Days 0, 3, 6, and 9 with harvest on Day 10. In this *Alternaria* adaptive immunity protocol, WT mice had a significant increase in total IgE, signifying the activation of the adaptive immune response. This was further supported by a significant increase in serum antigen-specific IgG1 in the WT mice challenged with the *Alternaria* adaptive immunity protocol, but not the 4-consecutive-day protocol. Serum antibody levels from naive mice are shown as a negative control.

IP Deficiency Increased the Number of Perivascular Eosinophils and Airway Mucus in Response to Airway *Alternaria alternata* Extract Challenge

Based on our finding that there was an increase in IL-5–expressing ILC2s in the

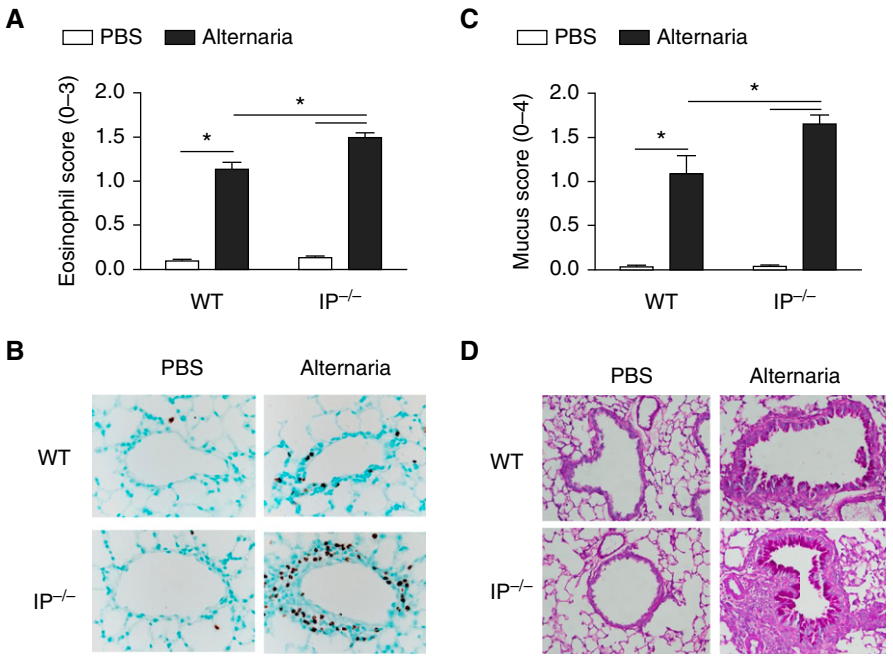

Figure 3. (*A*) Scoring of perivascular eosinophils in either phosphate-buffered saline (PBS)- or *Alternaria* extract–challenged wild-type (WT) or prostaglandin I_2 receptor–deficient ($IP^{-/-}$) mice 1 day after 4 consecutive days of intranasal challenge. (*B*) The lung sections were stained with anti–major basic protein antibody and were read by a pathologist masked to the treatment groups. n = 5 mice in each group. (*C*) Scoring of mucus in either PBS- or *Alternaria* extract–challenged WT or $IP^{-/-}$ mice 2 days after 4 consecutive days of intranasal challenge. (*D*) The lung sections were stained with periodic acid Schiff and were read by a pathologist masked to the treatment groups. n = 4 mice in each PBS-challenged group and n = 7 in each *Alternaria*-challenged group. *$P < 0.05$.

lungs of $IP^{-/-}$ mice challenged with intranasal *A. alternata* extract compared with WT mice, we hypothesized that there would be a greater number of perivascular eosinophils in $IP^{-/-}$ mice compared with WT mice following *Alternaria* extract challenge. To test this hypothesis, we assessed perivascular eosinophils by histopathology in mice harvested 1 day after 4 consecutive days of either intranasal PBS or *A. alternata* extract challenge (protocol shown in Figure E3). We found that there were virtually no perivascular eosinophils following intranasal PBS challenge, although there was a significant increase in perivascular eosinophils in WT mice intranasally challenged with *A. alternata* extract (Figure 3A). There was a further statistically significant increase in perivascular eosinophils in $IP^{-/-}$ mice challenged intranasally with *A. alternata* extract compared with similarly challenged WT mice. Representative histopathology sections stained with anti–major basic protein antibody are shown in Figure 3B. Because few IL-5–expressing $CD4^+$ T cells were present after 4 consecutive days of intranasal *Alternaria* extract challenge compared with significantly more ILC2s at the same point, this suggests, but does not confirm, that the increased number of IL-5–expressing ILC2s as a result of deficient IP signaling may be responsible for the increased perivascular eosinophils seen in the $IP^{-/-}$ mice compared with the WT mice.

Because there was an increase in lung IL-13–expressing ILC2s in the lungs of $IP^{-/-}$ mice, we further hypothesized that there is an increase in airway mucus in $IP^{-/-}$ mice compared with WT mice following *Alternaria* extract challenge. To test this hypothesis, we performed PAS staining on lung sections harvested 2 days following 4 consecutive days of either intranasal PBS or *A. alternata* extract challenge. We found that there was no PAS staining in airways following intranasal PBS challenge in WT or $IP^{-/-}$ mice, whereas there was a significant increase in PAS staining in WT and $IP^{-/-}$ mice intranasally challenged with *A. alternata* extract compared with PBS-challenged control mice (Figures 3C and 3D). There was a further statistically significant increase in PAS staining in airways in $IP^{-/-}$ mice challenged intranasally with *A. alternata* extract compared with similarly challenged WT mice.

Exogenous Administration of the PGI_2 Analog Cicaprost Decreased the Number of Mouse Lung ILC2s in Response to Airway *Alternaria* Extract Challenge

Our finding that endogenous PGI_2 decreased both the number of ILC2s and the expression of IL-5 and IL-13 by lung ILC2s in response to *Alternaria* extract challenge led us to hypothesize that an inhaled PGI_2 analog inhibits the number of airway *Alternaria* challenge–induced ILC2s. To test this hypothesis, we treated mice with either 50 μl PBS or 2.5 μg cicaprost in 50 μl PBS intranasally 15 minutes before each of the challenges with PBS or *Alternaria* extract on 4 consecutive days.

First, we measured IL-5 and IL-13 proteins by ELISA in WT mice 24 hours after 4 consecutive days of either PBS or *Alternaria* extract challenge. We found a statistically significant fourfold decrease in IL-5 and IL-13 protein expression in the BAL fluid (Figure 4A) of the mice pretreated with cicaprost compared with those pretreated with vehicle. This suggested that cicaprost was inhibiting the innate immune response to airway *Alternaria* extract challenge.

The number of $lin^-CD45^+CD25^+CD127^+IL\text{-}5^+$ cells and $lin^-CD45^+CD25^+CD127^+IL\text{-}13^+$ cells was determined following cell surface marker and intracellular cytokine staining in PBS- or *Alternaria* extract–challenged WT mice that were either pretreated with intranasally administered cicaprost or vehicle (*see* Figure E7). Although there was no change in the percentage of lung cells that were either IL-5– or IL-13–expressing ILC2s, there was a statistically significant approximate threefold decrease in the number of IL-5– and IL-13–expressing ILC2s in the *Alternaria* extract–challenged WT mice pretreated with cicaprost compared with those mice pretreated with vehicle (Figure 4B). There was no difference in the number of IL-5– and IL-13–expressing ILC2s in the PBS-challenged WT mice pretreated with cicaprost versus vehicle pretreatment. There was also a significant decrease in the MFI of IL-5 and IL-13 staining in

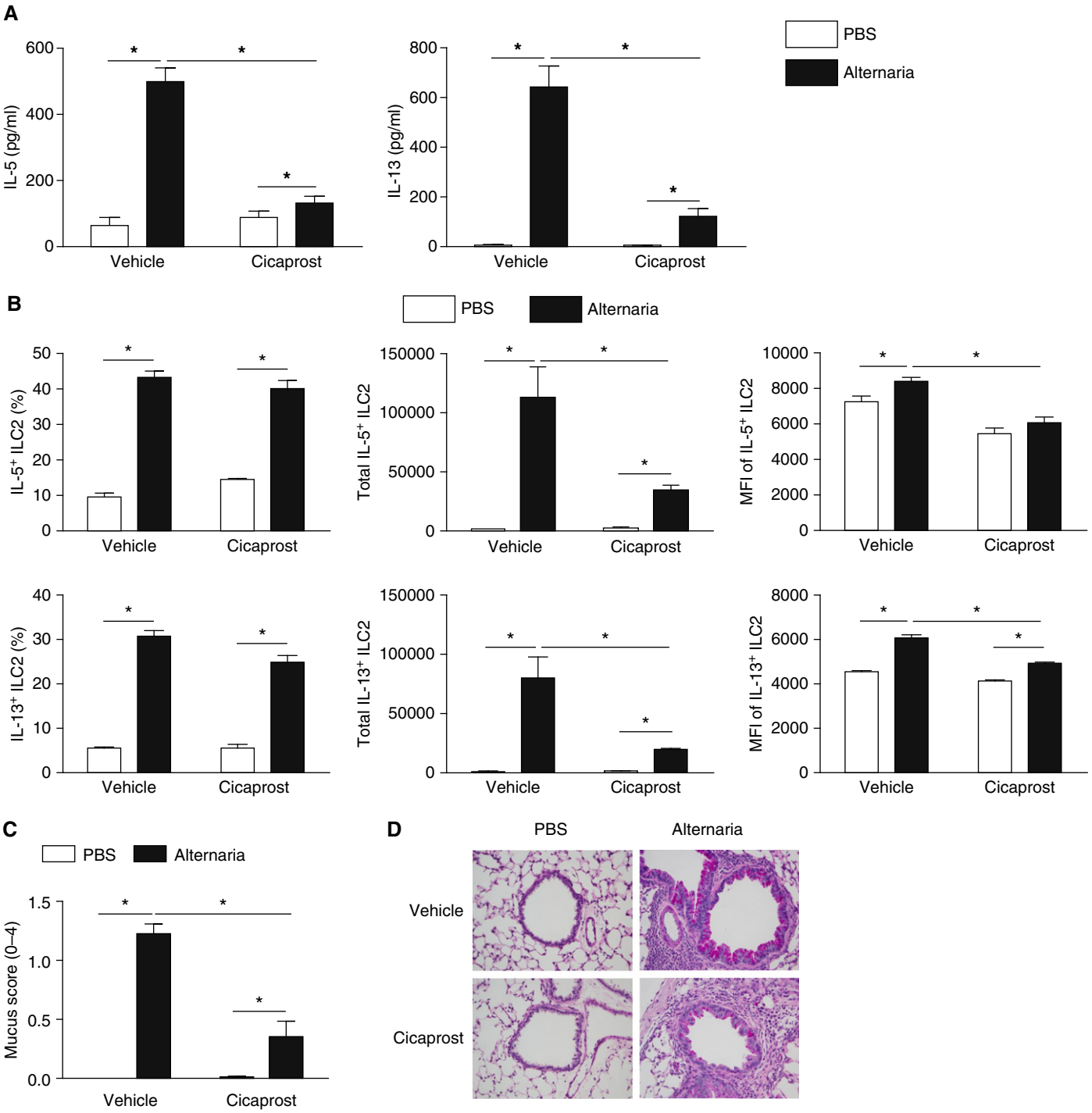

Figure 4. (*A*) IL-5 (*left*) and IL-13 (*right*) protein expression in bronchoalveolar lavage fluid from phosphate-buffered saline (PBS)- (n = 4) or *Alternaria* extract– (n = 6) challenged wild-type (WT) mice that were either treated with vehicle or cicaprost (2.5 μg) before the respective challenge. (*B*) The percentage, number, and IL-5 and IL-13 MFI of $lin^{-}CD45^{+}CD25^{+}CD127^{+}$ group 2 innate lymphoid cells (ILC2s) in PBS- (n = 4) or *Alternaria* extract– (n = 6) challenged WT and prostaglandin I_2 receptor–deficient mice. (*C*) Scoring of mucus in either PBS- or *Alternaria* extract–challenged WT mice treated with either vehicle or cicaprost 2 days after 4 consecutive days of intranasal challenge. (*D*) The lung sections were stained with periodic acid Schiff and were read by a pathologist masked to the treatment groups. n = 4 mice in each PBS-challenged group and n = 6 in each *Alternaria*-challenged group. *$P < 0.05$. MFI = mean fluorescence intensity.

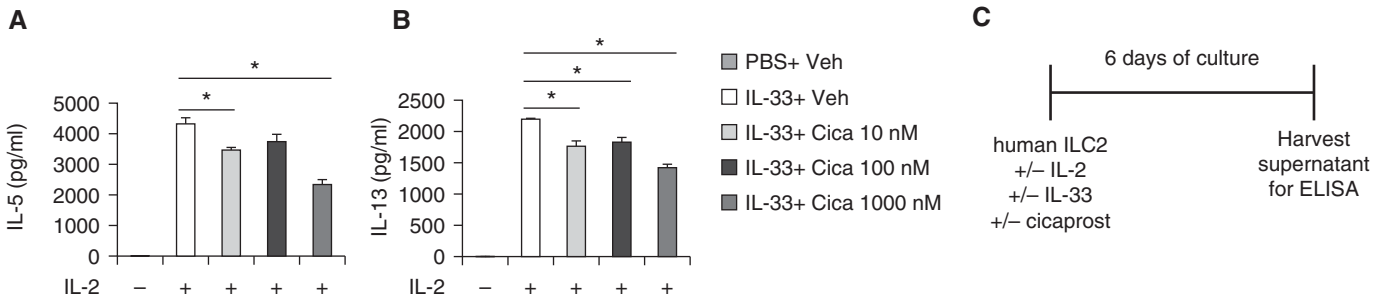

Figure 5. (*A* and *B*) The effect of IL-33 (10 ng/ml) and either vehicle or cicaprost (10, 100, and 1,000 nM) on IL-5 (*A*) and IL-13 (*B*) protein expression by human group 2 innate lymphoid cells (ILC2s) after 6 days of culture. $^*P < 0.05$. The data are representative of three separate experiments. (*C*) Time course of the experiment. Cica = cicaprost; PBS = phosphate-buffered saline; Veh = vehicle.

the lung ILC2s in the *Alternaria*-challenged WT mice pretreated with cicaprost compared with those that were pretreated with vehicle.

These results reveal that an inhaled PGI_2 analog significantly inhibited the number of ILC2s in response to *Alternaria* extract challenge, and also reduced IL-5 and IL-13 expression by lung ILC2s on a per cell basis in response to this same challenge. The total number of ILC2s in the *Alternaria* extract–challenged cicaprost-treated group was significantly decreased compared with the vehicle-treated group (*see* Figure E7B). ILC2s as a percentage of all lin^-CD45^+ cells are shown in Figure E7B. Because there was a decrease in the number of IL-13–expressing ILC2s in the lungs of cicaprost-treated WT mice compared with vehicle-treated mice, we hypothesized that there would be an inhibition of airway mucus in cicaprost-treated mice compared with WT mice following *Alternaria* extract challenge. To test this hypothesis, we performed PAS staining on lung sections harvested 2 days following 4 consecutive days of either intranasal PBS or *A. alternata* extract challenge. We found that there was a significant decrease in PAS staining in WT mice treated with cicaprost treatment before intranasal challenge with *A. alternata* extract compared with vehicle treatment (Figures 4C and 4D).

IL-5 and IL-13 Expression by Human ILC2s Was Inhibited by a PGI_2 Analog

Based on our *in vitro* data that cicaprost inhibits IL-5 and IL-13 protein expression by mouse ILC2s, and our *in vivo* data that endogenous PGI_2 and an exogenous PGI_2 analog inhibit *Alternaria* extract–induced mouse ILC2 production of these same cytokines, we hypothesized that cicaprost inhibits IL-5 and IL-13 expression by human ILC2s. To test this hypothesis, we first isolated human ILC2s from white blood cell filters obtained from the American Red Cross as a byproduct of blood donation. The gating strategy for isolation of ILC2s is shown in Figure E8. lin^- cells were isolated using a magnetic microbead kit. The cells were then sorted by flow cytometry to obtain $lin^-CD25^+CD127^+$ cells. Freshly sorted ILC2s were then cultured in IL-2 and IL-33 in the absence or presence of cicaprost at a concentration of 10, 100, or 1,000 nM for 6 days (Figure 5). IL-2 and IL-33 increased IL-5 and IL-13 protein expression by human ILC2s compared with cells cultured with vehicle. Cicaprost treatment significantly decreased ILC2 production of IL-5 at the 10- and 1,000-nM concentrations, but not at 100 nM. However, cicaprost treatment significantly decreased ILC2 production of IL-13 at 10-, 100-, and 1,000-nM concentrations. In addition, cicaprost at 100 and 1,000 nM significantly suppressed Id2 expression in human ILC2s (*see* Figure E9).

Discussion

In this manuscript, we report that mouse ILC2s express the PGI_2 receptor IP and that the PGI_2 analog cicaprost significantly inhibited IL-33–induced expression of IL-5 and IL-13, cytokines critical for the allergic phenotype, and also blunted ILC2 proliferation, likely through its inhibition of ILC2 mRNA expression of Id2. In addition, endogenous PGI_2 signaling through IP inhibited lung IL-5 and IL-13 protein expression following airway *Alternaria* extract challenge and also inhibited the number of lung ILC2s and the MFI of IL-5 and IL-13, suggesting the PGI_2 reduces the production of these cytokines on a per cell basis. Endogenous PGI_2 also inhibited the number of perivascular eosinophils induced by *Alternaria* extract challenge, presumably as a result of the inhibition of ILC2 function and number because there were very few IL-5-expressing $CD4^+$ cells at this time point. Administration of a PGI_2 analog by inhalation similarly inhibited lung IL-5 and IL-13 protein expression following airway *Alternaria* extract challenge. In contrast to endogenous PGI_2 signaling, the PGI_2 analog did not alter the percentage of lung ILC2s that expressed IL-5 or IL-13, nor did cicaprost decrease the MFI of IL-5 and IL-13 staining; however, cicaprost did significantly reduce the total number of ILC2s in the lung that expressed IL-5 and IL-13 protein. Finally, the PGI_2 analog cicaprost significantly inhibited IL-33–induced expression of IL-5 and IL-13 from human ILC2s.

This is the first report of a PG inhibiting ILC2 function. The prostanoids have diverse effects on the inflammatory cascade and there is strong evidence that they have a role in asthma pathogenesis (38, 39). PGD_2, the major PG produced by mast cells, increased IL-13 expression by human ILC2s and synergistically augmented expression of this cytokine by ILC2s in response to the epithelial-derived cytokines IL-25 and IL-33 (40). Leukotrienes are also products of arachidonic metabolism, formed by the action of 5-lipoxygenase (39). The cysteinyl leukotrienes, LTC_4, LTD_4, and LTE_5, are expressed in the lung of subjects with asthma as a result of allergen challenge and signal through the cysteinyl leukotriene receptor 1 (cysLTR1) (38). ILC2s express cysLTR1, and LTD_4 potently induces

cysLTR1-dependent ILC2 production of IL-5 and IL-13 (41). In addition, LTD_4 potentiated *Alternaria* extract–induced ILC2 proliferation and accumulation (41). With the exception of our report that PGI_2 inhibits ILC2 function, the only other lipid mediator that has been reported to inhibit ILC2 function is lipoxin A_4, which decreased IL-13 release by human peripheral blood ILC2s (40).

In this project, we focused on IL-33 as an inducer of ILC2 cytokine production. IL-33 is constitutively expressed in the nucleus of epithelial and endothelial cells, and is released on cell injury as an alarmin (42). Proteases disrupt mucosal integrity by digesting cell adhesion molecules, act on protease-activated receptors to activate epithelial cells, and can induce IL-33 release by airway epithelial cells (26). As a result of being expressed by cells that interface with the environment, IL-33 functions as an early initiator of inflammation. In addition, IL-33 expression is stimulated in inflamed tissues and can further amplify inflammatory responses. IL-33 has been implicated as a pathogenic factor in several inflammatory states including asthma, arthritis, ulcerative colitis, and fibrotic diseases. Although IL-33, IL-25, and thymic stromal lymphopoietin are all expressed by epithelial cells in response to proteases, a recent study reveal that IL-33 is the most important cytokine that drives Th2-type cytokine expression by lung ILC2s (26).

We found that IL-33 induced the proliferation of WT and $IP^{-/-}$ ILC2s *in vitro* to a similar degree. Importantly, the proliferation of WT ILC2s was inhibited by cicaprost, whereas there was no effect of cicaprost on IL-33–induced proliferation of $IP^{-/-}$ ILC2s. This is a crucial finding because it reveals that the effect of cicaprost on IL-33–induced proliferation was a receptor-mediated suppression of proliferation. The number of ILC2s that we could isolate from the bone marrow was very limited and although proliferation was induced by IL-33, cicaprost treatment reduced this number dramatically. This decrease in ILC2 number in the cicaprost-treated group prevented us from being able to perform either real-time PCR or flow cytometry to assess transcription factors involved in either protection against or induction of apoptosis. PGI_2 protected against endothelial cell apoptosis induced by cigarette smoke, H_2O_2-induced apoptosis of vascular smooth muscle cells, and bleomycin-induced apoptosis of lung cells (43–45). However, the effect of PGI_2 on proliferation and apoptosis of lymphoid cells has not previously been published to the best of our knowledge.

Studies performed by our laboratory and other investigators reveal that PGI_2 is a critical inhibitor of allergic lung inflammation and a potential target for the treatment of allergic diseases, such as asthma. Eosinophils are potent effector cells in allergic inflammation (46, 47), and PGI_2 inhibits eosinophil chemotaxis. For instance, PGI_2 inhibited the mobilization and migration of bone marrow eosinophils in guinea pigs (47). In *in vitro* studies, PGI_2 blocked the adhesion of eosinophils to fibronectin and the rapid up-regulation and activation of the adhesion molecule CD11b. Furthermore, when endothelial cells were treated with a COX inhibitor to prevent PGI_2 production, adhesion of eosinophils to endothelial monolayers and resultant transendothelial migration were markedly augmented. In addition, an IP antagonist increased eosinophil adhesion to endothelial cells (48). In the ovalbumin model, $IP^{-/-}$ mice had significantly augmented pulmonary proinflammatory Th2 cytokine expression, airway mucus, and airway responsiveness compared with WT mice in both acute and chronic allergen-induced inflammation models (17, 18, 49).

Supporting these findings, our group reported that the PGI_2 analogs iloprost and cicaprost significantly reduced LPS-induced DC expression of proinflammatory cytokines and chemokines in an IP-specific fashion, while increasing the production of the antiinflammatory cytokine IL-10 by DCs *in vitro* (19). In these experiments we used PGI_2 analogs, iloprost and cicaprost, which have a much longer half-life of 20–30 minutes than the half-life of PGI_2, which is 60–90 seconds (36). Iloprost and cicaprost also suppressed LPS-induced expression of CD86, CD40, and major histocompatibility complex class II molecules by DCs and inhibited the ability of DCs to stimulate antigen-specific $CD4^+$ T-cell proliferation and production of the Th2 cytokines IL-5 and IL-13 (19). We also reported that PGI_2 analogs inhibited the ability of $CD4^+$ T cells activated in the Th2 polarizing conditions to express IL-4 and IL-13 *in vitro* (20). PGI_2 is also produced by follicular DCs and inhibited T-cell proliferation (50).

These results suggest that exogenous PGI_2 decreases allergic inflammatory responses *in vivo* and this was confirmed in the mouse model when iloprost inhibited the maturation and migration of lung DCs to the mediastinal LNs, resulting in decreased induction of an allergen-specific Th2 response in these nodes (14). In this *in vivo* model, iloprost-treated DCs down-regulated Th2 differentiation from naive T cells and were also unable to boost effector cytokine production in primed Th2 cells (14). Furthermore, PGI2 was critical to immune tolerance induced by mucosal exposure to antigen in an mouse model of allergic airway inflammation (49). Although these studies revealed that PGI_2 has important immunomodulatory effects on DCs and T cells, our novel results indicate that PGI_2 has profound effects on ILC2 cytokine protein expression and the number of ILC2s in the lung following acute allergen-challenge without sensitization.

The results of our study are important for two reasons. First, investigating the role of endogenous PGI_2 is critical to understand the effect of COX-inhibiting drugs, one of the most widely used classes of over-the-counter medications in the world, on innate allergic immune responses. Second, investigating the role of exogenous PGI_2 is critical to determine the potential effects of inhaled PGI_2 or its analogs on the innate allergic immune response because such agents are currently used therapeutically for pulmonary hypertension (36), and could be potentially used for allergic respiratory diseases, such as asthma. The results we obtained from our *in vivo* experiments are likely generalizable to other common allergens, such as dust mites and cockroach, which also have high levels of protease activity similar to *A. alternata*. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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