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Prostaglandin I₂ Suppresses Proinflammatory Chemokine Expression, CD4 T Cell Activation, and STAT6-Independent Allergic Lung Inflammation

Weisong Zhou,* Jian Zhang,* Kasia Goleniewska,* Daniel E. Dulek,* Shinji Toki,* Dawn C. Newcomb,* Jacqueline Y. Cephus,* Robert D. Collins,^{†,1} Pingsheng Wu,* Mark R. Boothby,[†] and R. Stokes Peebles, Jr.*[†]

Allergic airway diseases are immune disorders associated with heightened type 2 immune responses and IL-5 and IL-13 production at the site of inflammation. We have previously reported that cyclooxygenase (COX) inhibition by indomethacin augmented allergic airway inflammation in a STAT6-independent manner. However, the key COX product(s) responsible for restraining indomethacin-mediated STAT6-independent allergic inflammation is unknown. In this study, using the mouse model of OVA-induced allergic airway inflammation, we identified that PGI₂ receptor (IP) signaling was critical for indomethacin-induced, STAT6-independent proallergic effects. We demonstrated that IP deficiency increased inflammatory cell infiltration, eosinophilia, and IL-5 and IL-13 expression in the lung in a STAT6-independent manner. The augmented STAT6-independent allergic inflammation correlated with enhanced primary immune responses to allergic sensitization and elevated production of multiple inflammatory chemokines (CCL11, CCL17, CCL22, and CXCL12) in the lung after allergen challenge. We also showed that the PGI₂ analogue cicaprost inhibited CD4 T cell proliferation and IL-5 and IL-13 expression in vitro, and IP deficiency diminished the stimulatory effect of indomethacin on STAT6-independent IL-5 and IL-13 responses in vivo. The inhibitory effects of PGI₂ and the IP signaling pathway on CD4 T cell activation, inflammatory chemokine production, and allergic sensitization and airway inflammation suggest that PGI₂ and its analogue iloprost, both Food and Drug Administration–approved drugs, may be useful in treating allergic diseases and asthma. In addition, inhibiting PGI₂ signaling by drugs that either block PGI₂ production or restrain IP signaling may augment STAT6-independent pathways of allergic inflammation. *The Journal of Immunology*, 2016, 197: 1577–1586.

Allergic diseases are immune disorders associated with heightened type 2 immune responses at the site of inflammation. STAT6 is activated by both IL-4 and IL-13, and plays an important role in Th2 cell differentiation and type 2 immune responses (1). We have previously shown that the cyclooxygenase (COX) inhibitor indomethacin augmented allergic Th2 cytokine responses and lung inflammation in a STAT6-independent manner (2, 3). However, which COX product(s) suppressed by COX inhibition is critical for indomethacin-induced, STAT6-independent proallergic effect has not been identified.

The COX pathway of arachidonic acid metabolism is responsible for the formation of PGD₂, PGE₂, PGF_{2α}, PGI₂, and thromboxane A₂, and has immunoregulatory functions in the development and manifestation of allergic diseases (2–11). COX-inhibiting drugs such as indomethacin and the COX-2 selective inhibitor NS-398 increased allergic inflammation in the airway and skin in mice (9, 12), suggesting that some lipid products formed in the COX pathway have inhibitory effects on allergic diseases. PGI₂ functions as an immune modulator through the PGI₂ receptor (IP) expressed on immune cells such as dendritic cells, T cells, and alveolar macrophages (13–15). Multiple studies indicate that PGI₂ suppressed Th2 immune responses and allergic inflammation (10, 11, 13, 14, 16, 17). Although the suppressive effect of PGI₂ and its analogues on Th2 immune responses and allergic inflammation is known, the role of PGI₂ in STAT6-independent allergic inflammation has not been reported.

Chemokines are small chemoattractant proteins and critical mediators for immune and inflammatory cell trafficking in allergic inflammation. Chemokines exert their biological functions on distinct subsets of cells. For example, CCL2 (also known as monocyte chemoattractant protein-1, MCP-1) attracts monocytes, dendritic cells, and memory T cells to the site of its release (18, 19). CCL11 (also known as eotaxin-1) recruits eosinophils, one of the hallmarks of allergic inflammation (20). CXCL12 (also known as stromal cell-derived factor 1, SDF-1) is strongly chemotactic to lymphocytes (21, 22). CCL17 (also known as the thymus and activation-regulated chemokine, TARC) and CCL22 (also known as macrophage-derived chemokine, MDC) attract Th2 cells via binding to CCR4 (23). Chemokines are responsible for the cellular composition at inflammatory sites. Studies have shown that the COX pathway and its downstream

*Division of Allergy, Pulmonary, and Critical Care Medicine, Department of Medicine, Vanderbilt University School of Medicine, Nashville, TN 37232; and [†]Department of Pathology, Microbiology, and Immunology, Vanderbilt University School of Medicine, Nashville, TN 37232

¹Deceased.

ORCID: 0000-0003-4379-9118 (S.T.); 0000-0002-1429-7875 (R.S.P.).

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Address correspondence and reprint requests to Dr. Weisong Zhou, Division of Allergy, Pulmonary and Critical Care Medicine, Department of Medicine, Vanderbilt University School of Medicine, T-1217 MCN, 21st Avenue South, Nashville, TN 37232. E-mail address: weisong.zhou@vanderbilt.edu

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Abbreviations used in this article: alum, aluminum hydroxide; BALF, bronchoalveolar lavage fluid; COX, cyclooxygenase; DKO, double knockout; IP, PGI₂ receptor; KO, knockout; WT, wild type.

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lipid mediators regulate chemokine expression in inflammation. Inhibition of COX-2 augmented CCL3 (MIP-1 α) and CCL5 (RANTES) expression by *Leishmania*-infected peritoneal macrophages (24). PGE₂ attenuated LPS-induced mRNA and protein expression of CXCL-10 (IP-10) and CCL3 in mouse adipose tissue (25). PGI₂ analogues suppressed LPS-induced CCL2 and CCL3 production in human monocytes via IP and the cAMP pathway (26, 27), and inhibited CXCL9 (MIG) and CXCL10 release by human airway epithelial cells (28). However, it is not clear whether PGI₂ regulates the expression of chemokines that are critical for the development of allergic inflammation in the lung.

In this study, we focused on the regulation of STAT6-independent Th2 immune responses and allergic inflammation by the COX pathway and PGI₂. We hypothesized that PGI₂ and the IP signaling pathway decrease allergen-induced proinflammatory chemokine expression and inhibit STAT6-independent allergic airway inflammation. We generated mice that are deficient in both STAT6 and IP (IP-STAT6 double-knockout [DKO] mice) and used the mice in the OVA-induced allergic airway inflammation model. We found that deficiency in the IP signaling pathway increased STAT6-independent proinflammatory chemokine expression and primary CD4 T cell responses in the spleen during immune sensitization, and augmented chemokine production and inflammatory responses in the lung after OVA challenge. We also demonstrated that indomethacin augmented STAT6-independent allergic airway responses by inhibiting the PGI₂/IP signaling pathway.

Materials and Methods

Mice

Wild type (WT) BALB/c mice and STAT6 KO mice on a BALB/c background were obtained from Jackson Laboratory (Bar Harbor, ME). IP KO mice on a C57BL/6 background were generated by homologous recombination in embryonic stem cells and kindly provided by Dr. Garret FitzGerald at the University of Pennsylvania (29). The IP KO mice were backcrossed to a BALB/c background for 10 generations. IP-STAT6 DKO mice were generated by breeding STAT6 KO mice with IP KO mice and subsequent genotyping. Age-matched WT, IP KO, STAT6 KO, and IP-STAT6 DKO mice were used at 8–12 wk old. Animal experiments were reviewed, approved by the Institutional Animal Care and Use Committee at Vanderbilt University, and conducted according to the guidelines for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources, National Research Council.

Induction of allergic airway inflammation

WT, IP KO, STAT6 KO, and IP-STAT6 DKO mice were sensitized with an i.p. injection of 100 μ l of OVA solution containing 10 μ g of OVA (chicken OVA, grade V; Sigma-Aldrich) formulated with 20 mg of aluminum hydroxide (alum) in PBS on day 0 (Fig. 1A). On days 14–17, the mice were exposed to aerosols of 1% OVA-PBS solution created by an ultrasonic nebulizer for 40 min per day. On day 18, bronchoalveolar lavage fluid (BALF) was collected and mouse lungs were harvested (Fig. 1A). In COX inhibition experiments, mice were provided drinking water containing indomethacin (45 μ g/ml) or vehicle (ethanol) from day -2 (2 d before OVA-alum injection) to day 2 (2 d after OVA-alum injection).

Histological analyses of lung sections

The mice were sacrificed on day 18 and the lung block was removed. Lung tissue was fixed in 10% formalin solution, paraffin embedded, cut in 6- μ m sections, mounted, and stained with periodic acid–Schiff to assess mucus.

Spleen cell culture

Spleens were harvested from either naive mice or OVA-sensitized mice at day 5 after OVA/alum i.p. injection (Fig. 1A) and ground through 70- μ m cell strainers for preparation of single-cell suspension. RBCs were lysed and the spleen cells were cultured in 96-well flat-bottom plates with BSA (100 μ g/ml, as control protein stimulation) or OVA (100 μ g/ml) for 3 d. The LPS concentrations in BSA- or OVA-containing culture media were

<0.123 pg/ml as determined by an ELISA kit (Biomatik, Atlanta, GA). The culture supernatant was harvested for determination of IL-5 and IL-13 levels.

CD4 T cell culture and flow cytometry

CD4 T cells were purified from the splenic cells of WT BALB/c mice by a mouse naive CD4 T cell isolation kit (Miltenyi Biotec, Auburn, CA). The purified CD4 T cells (purity >94%, as assessed by flow cytometry) were resuspended at 1×10^6 cells/ml in RPMI 1640 medium (Mediatech, Herndon, VA) supplemented with 10% FBS (HyClone, Logan, UT), 4 mM of L-glutamine, 1 mM of sodium pyruvate, 55 μ M of 2-ME, 10 mM of HEPES, 100 U/ml penicillin, and 100 μ g/ml streptomycin. The cells were stimulated with plate-bound anti-CD3 (5 μ g/ml) and anti-CD28 (1 μ g/ml; BD Biosciences, San Diego, CA) in 96-well flat-bottom plates and treated with cicaprost at various concentrations or vehicle for 3 d. In T cell proliferation experiments, naive CD4 T cells were stained with CFSE, followed by cell culture with plate-bound anti-CD3 and anti-CD28 and treatment with cicaprost (a generous gift provided by Dr. Manuela Huebner, Bayer HealthCare, Berlin, Germany) or vehicle. After 3 d of cell culture, CFSE intensity of the cells was determined by flow cytometry gated for all cells with LSR II flow cytometer (BD Biosciences). The culture supernatant was harvested for multiple cytokine ELISA assays. T cells were also harvested at day 2 for RT-PCR analyses of Bcl6, STAT1, STAT6, and β -actin mRNA expression.

Cytokine, chemokine, and OVA-specific IgE measurements by ELISA

To measure the levels of IL-2, IL-4, IL-5, IL-13, CCL2, CCL11, CCL17, CCL22, CXCL9, CXCL10, CXCL12, IL-1 α , IL-1 β , IL-6, TNF- α , and IFN- γ in the cell culture supernatant, BALF, and lung homogenate, we used Quantikine and DuoSet ELISA kits from R&D Systems according to the manufacturer's instructions. OVA-specific IgE was measured by an ELISA kit from Biolegend. Measurements below the limit of detection were assigned a value of half the lower limit of detection for purposes of statistical analyses.

Real-time PCR

The probes and primers of Bcl6 (Mm0047633_m1), STAT1 (Mm00439531_m1) and STAT6 (Mm01160477_m1), and β -actin (Mm00607939_s1) were purchased from Applied Biosystems (Foster City, CA). RT-PCR was performed following TaqMan Gene Expression Assay Protocol provided by Applied Biosystems.

Statistical analysis

The results were presented as mean $\pm$ SEM. Statistical analyses were conducted by using one-way ANOVA with a Bonferroni post hoc test.

Results

IP deficiency increased OVA-induced, STAT6-independent allergic inflammation in the lung

We have previously published that the proallergic effect of COX inhibition by indomethacin was independent of the STAT6 signaling pathway, suggesting that lipid products formed in the COX pathway inhibit STAT6-independent allergic inflammation (2). Because of the immune-suppressive effects of PGI₂ on Th2 immune responses and allergic inflammation (10, 11, 13, 14, 16, 17, 30), we hypothesized that PGI₂ inhibits STAT6-independent allergic airway inflammation. To test this hypothesis, we generated IP-STAT6 DKO mice and induced OVA-specific allergic lung inflammation in IP-STAT6 DKO mice and in control STAT6 KO, IP KO, and WT mice by OVA/alum sensitization followed by OVA challenges (Fig. 1A). Mouse BALF was harvested 16 h after the last OVA challenge. We found that IP-STAT6 DKO mice had significantly augmented IL-5, IL-13, IL-1 α , and IL- β protein expression, but not IL-4, IL-6, and TNF- α expression, compared with STAT6 KO mice (Fig. 1). IP-STAT6 DKO also had increased numbers of total cells, eosinophils, and macrophages (Fig. 2) in BALF compared with STAT6 KO mice, indicating that IP deficiency activated STAT6-independent allergic

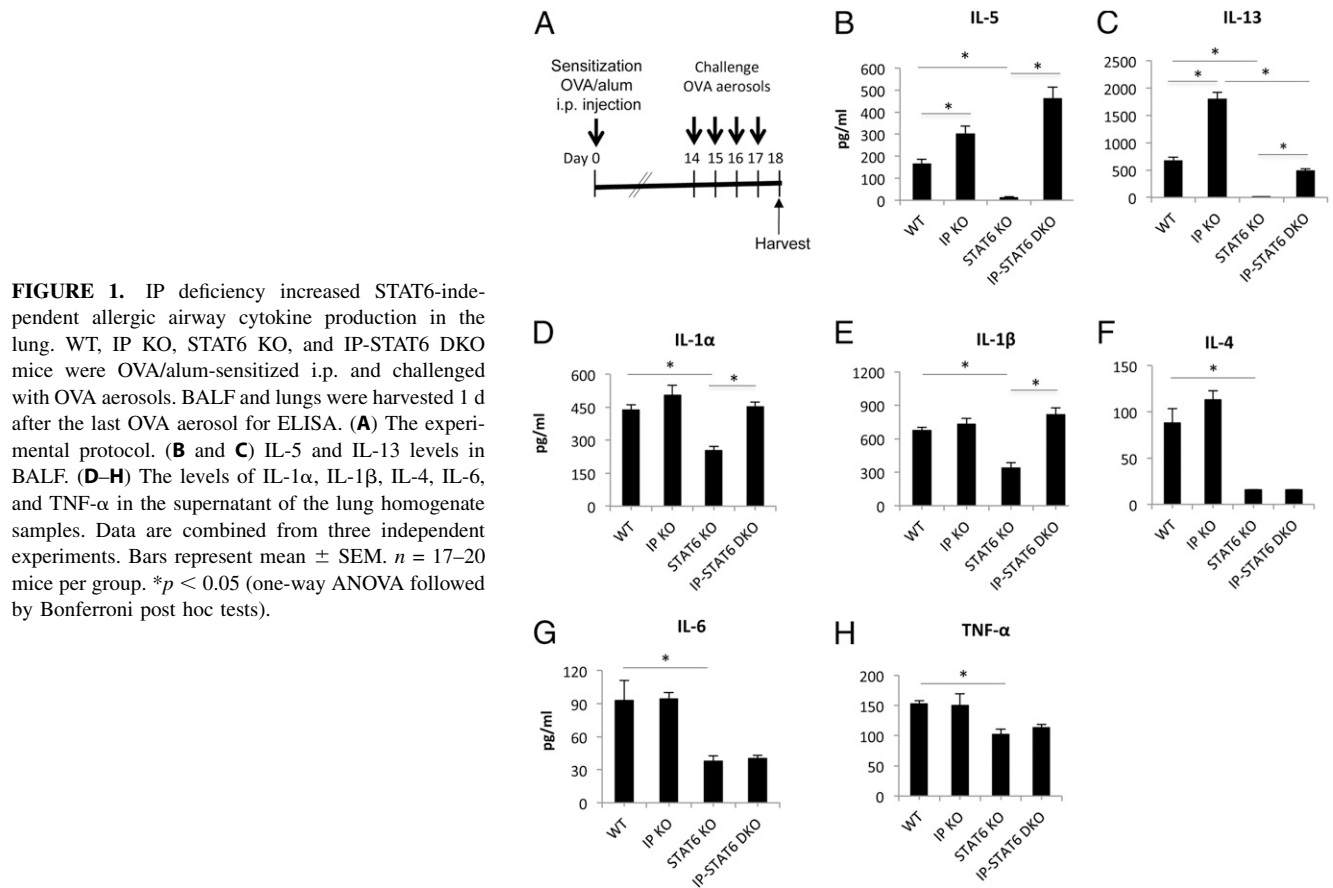


FIGURE 1. IP deficiency increased STAT6-independent allergic airway cytokine production in the lung. WT, IP KO, STAT6 KO, and IP-STAT6 DKO mice were OVA/alum-sensitized i.p. and challenged with OVA aerosols. BALF and lungs were harvested 1 d after the last OVA aerosol for ELISA. (A) The experimental protocol. (B and C) IL-5 and IL-13 levels in BALF. (D–H) The levels of IL-1 α , IL-1 β , IL-4, IL-6, and TNF- α in the supernatant of the lung homogenate samples. Data are combined from three independent experiments. Bars represent mean $\pm$ SEM. $n = 17$ –20 mice per group. * $p < 0.05$ (one-way ANOVA followed by Bonferroni post hoc tests).

lung inflammation. In contrast, STAT6 KO mice had neither detectable IL-13 protein (Fig. 1C) nor eosinophilia (Fig. 2B) in BALF. We found that IP KO mice developed heightened IL-5 and IL-13 responses (Fig. 1B, 1C) and had increased numbers of total cells, eosinophils, macrophages, and lymphocytes in BALF compared with WT mice (Fig. 2), consistent with previous studies by others (16, 17). IP-STAT6 DKO and STAT6 KO mice did not produce mucus in the airway after OVA sensitization and challenge, whereas WT and IP KO mice did (Supplemental Fig.

1), indicating that STAT6 is required for epithelial cell mucous production as reported by others (1).

IP deficiency increased STAT6-independent inflammatory chemokine responses in the lung

Jaffar and colleagues (10) reported that PGI₂ decreased OVA-induced allergic lung inflammation by inhibiting CD4 T cell recruitment. By extension, we hypothesized that the STAT6-independent allergic lung inflammation and Th2 cytokine responses

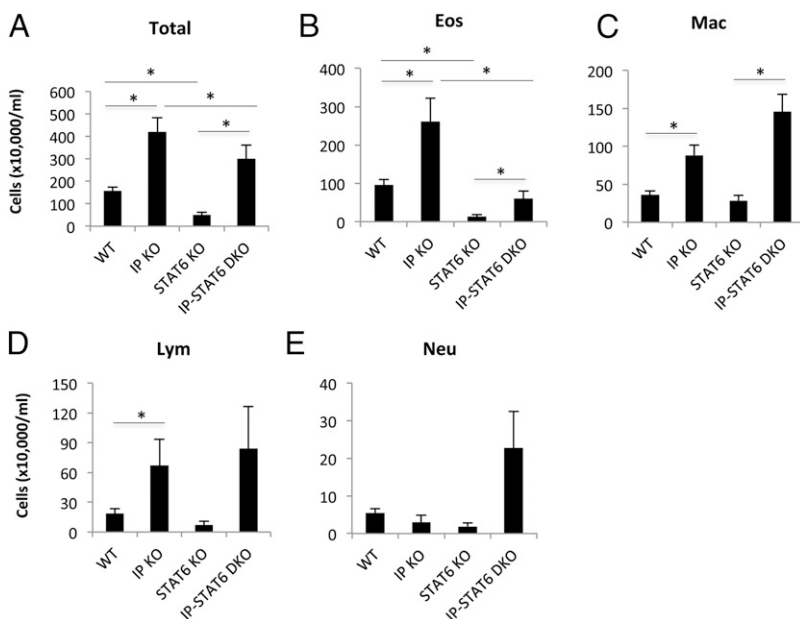


FIGURE 2. IP deficiency increased STAT6-independent allergic airway inflammatory cell infiltration in the lung. WT, IP KO, STAT6 KO, and IP-STAT6 DKO mice were OVA/alum-sensitized i.p. and challenged with OVA aerosols. BALF was harvested for total and differential cell counts. (A) Total cells. (B) Eosinophils. (C) Macrophages. (D) Lymphocytes. (E) Neutrophils. Data are combined from three independent experiments. Bars represent mean $\pm$ SEM. $n = 17$ –20 mice per group. * $p < 0.05$ (one-way ANOVA followed by Bonferroni post hoc tests).

in IP-STAT6 DKO mice resulted from increased chemokine responses in the lung. To test this hypothesis, we first determined whether IP deficiency causes constitutive changes of chemokine expression in naive mice. We measured chemokine levels in the lung homogenate of naive WT, IP KO, STAT6 KO, and IP-STAT6 DKO mice. We found that naive IP-STAT6 DKO mice had significantly higher levels of CCL11 than naive STAT6 KO mice (Supplemental Fig. 2). The levels of CCL2, CCL17, CCL22, and CXCL12 in the lung were similar between naive IP-STAT6 DKO mice and naive STAT6 KO mice (Supplemental Fig. 2). In naive IP KO mice, there were significantly higher levels of CCL2, CCL11, CCL22, and CXCL12 in the lung compared with those of naive WT mice (Supplemental Fig. 2). These data suggest that endogenous PGI₂ and IP signaling inhibit the expression of CCL2, CCL11, CXCL12, and CCL22 in STAT6-sufficient mice, but suppress only CCL11 expression in STAT6-deficient mice.

Next, we sensitized and challenged WT, IP KO, STAT6 KO, and IP-STAT6 DKO mice with OVA. Mouse lungs were harvested 16 h after the last OVA challenge and we determined the levels of chemokines that are involved in allergic inflammation in the lung homogenate. We found that IP-STAT6 DKO mice with OVA-induced allergic airway inflammation had increased levels of CCL11, CCL17, CCL22, CXCL9, and CXCL12 in the lung compared with OVA-sensitized and -challenged STAT6 KO mice (Fig. 3). These chemokine levels correlated with the augmented infiltration of eosinophils and macrophages in the lungs of IP-STAT6 DKO mice (Fig. 2). IP KO mice had greater levels of CCL11, CCL22, and CXCL12 in the lung than those of WT mice (Fig. 3), which correlated with increased allergic airway inflam-

mation in IP KO mice compared with WT mice (Fig. 2). These data indicate that IP deficiency activated chemokine responses and allergic airway inflammation in a STAT6-independent manner. Notably, compared with IP KO mice, IP-STAT6 DKO mice had significantly lower levels of CCL11, CCL17, and CCL22 expression in the lung (Fig. 3) and IL-13 expression (Fig. 1C) in BALF, and fewer numbers of total cells and eosinophils in the BALF (Fig. 2A, 2B), suggesting that the STAT6-dependent pathway contributes to the allergic airway inflammation in IP KO mice.

IP deficiency increased STAT6-independent immune sensitization in the spleen

We have previously shown that treating mice with the COX inhibitor indomethacin during immune sensitization promoted both the primary and the memory responses in OVA-induced allergic airway inflammation (2). We also demonstrated that the proallergic effect of indomethacin is dependent on CD4 T cells (3). To test whether IP deficiency increases STAT6-independent immune sensitization to OVA-specific CD4 T cell immune responses in the OVA model, we first characterized the primary immune responses after OVA sensitization in WT, IP KO, STAT6 KO, and IP-STAT6 DKO mice. We harvested mouse spleens 5 d after OVA/alum i.p. injection. We found that OVA/alum injection induced splenomegaly in IP-STAT6 DKO mice (Fig. 4A). IP-STAT6 DKO mice had significant increases in spleen weights compared with STAT6 KO mice (Fig. 4B). OVA-sensitized IP-STAT6 DKO mice also had increased numbers of total nucleated cells, eosinophils, and neutrophils in the spleen compared with OVA-sensitized STAT6 KO

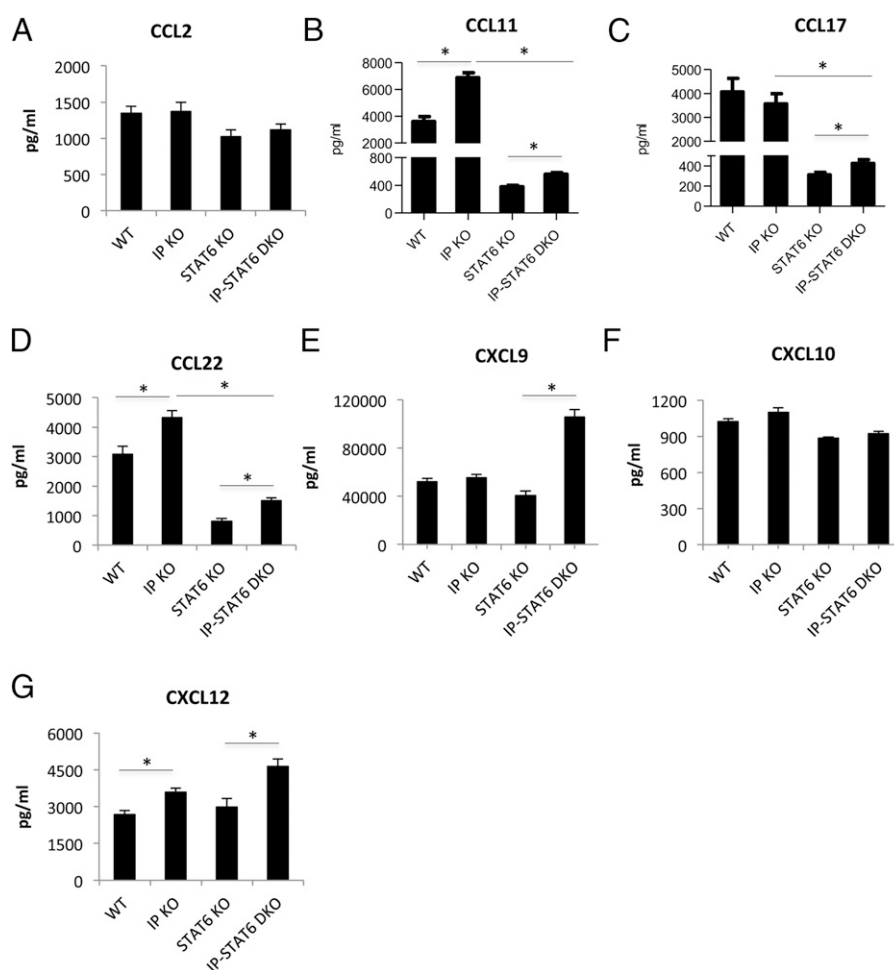


FIGURE 3. IP deficiency increased STAT6-independent proinflammatory chemokine production in the lung. WT, IP KO, STAT6 KO, and IP-STAT6 DKO mice were OVA/alum-sensitized i.p. and challenged with OVA aerosols. Mouse lungs were harvested, and the levels of chemokines in the supernatant of the lung homogenate samples were determined by ELISA. Data are combined from three independent experiments. (A) CCL2, (B) CCL11, (C) CCL17, (D) CCL22, (E) CXCL9, (F) CXCL10, (G) CXCL12. Bars represent mean $\pm$ SEM. $n = 17$ –20 mice per group. * $p < 0.05$ (one-way ANOVA followed by Bonferroni post hoc tests).

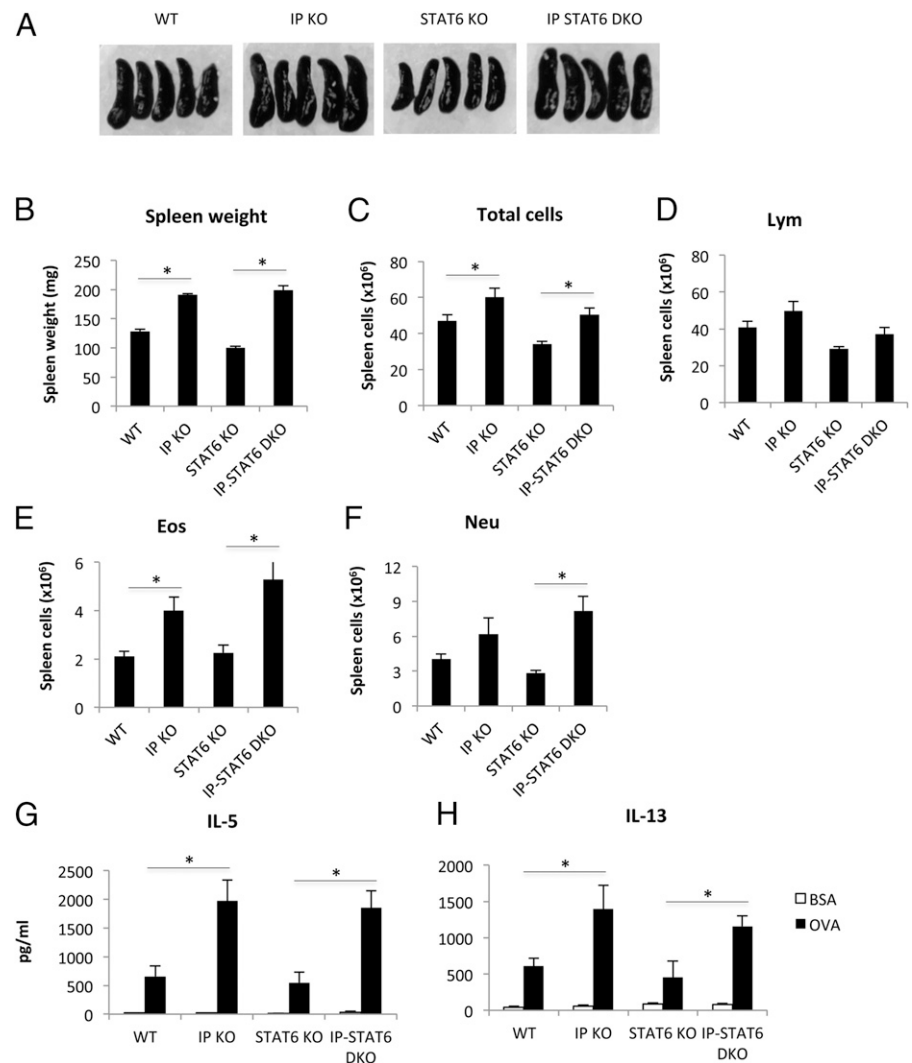


FIGURE 4. IP deficiency increased STAT6-independent primary immune responses to OVA/alum sensitization. Mice were i.p. injected with OVA/alum. Spleens were harvested at 5 d after the injection. **(A)** Spleen images. **(B)** Spleen weights. **(C)** The total numbers of RBC-depleted nucleated cells in the spleen. **(D–F)** RBC-depleted spleen cells stained for differential cell counts. **(G and H)** The spleen cells were cultured with BSA or OVA protein for 3 d. The levels of IL-5 and IL-13 in the cell culture supernatant were determined by ELISA. Results are combined data of three independent experiments. Bars represent mean $\pm$ SEM in (B)–(H). $n = 15$ mice per group. * $p < 0.05$ (one-way ANOVA followed by Bonferroni post hoc tests).

mice (Fig. 4C–F). Similarly, IP KO mice had increased numbers of total nucleated cells and eosinophils in the spleen compared with WT mice (Fig. 4C–E). Macrophages were not observed during the spleen cell counts. To further characterize the primary immune response to OVA sensitization, we stimulated RBC-depleted spleen cells in vitro with OVA protein or BSA protein as control for 3 d. We found that OVA, but not BSA, induced IL-5 and IL-13 responses by the spleen cells (Fig. 4G, 4H), indicating that the Th2 cytokine responses were OVA specific. IP-STAT6 DKO spleen cells produced greater amounts of IL-5 and IL-13 than STAT6 KO mice (Fig. 4G, 4H), suggesting that IP deficiency increased OVA-specific CD4 T cell activation and cytokine production in a STAT6-independent manner. Similarly, IP KO spleen cells produced more IL-5 and IL-13 in the culture supernatant than WT cells (Fig. 4G, 4H). These results suggest that endogenous PGI₂ and the IP signaling suppressed CD4 T cell activation and Th2 differentiation.

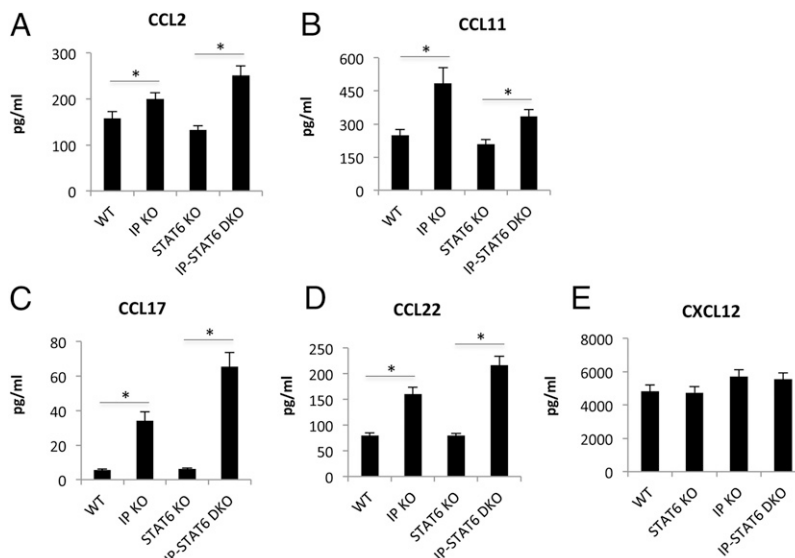
To determine whether IP deficiency causes constitutive splenomegaly, we harvested spleens of naive WT, IP KO, STAT6 KO, and IP-STAT6 DKO mice. We found that the spleen weights of naive IP-STAT6 DKO mice were 12% greater than those of naive STAT6 KO mice (Supplemental Fig. 3). However, the total numbers of nucleated cells in the spleen were not significantly different between IP-STAT6 DKO and STAT6 KO mice (Supplemental Fig. 3). Similarly, the spleen weights of naive IP KO mice were greater (15% more) than those of WT mice, but the total nucleated spleen cell

numbers were similar (Supplemental Fig. 3). These data indicate that IP deficiency caused slight splenomegaly but did not increase the total numbers of nucleated cells in the spleen.

IP deficiency increased STAT6-independent inflammatory chemokine responses for eosinophil recruitment in the spleen

IP-STAT6 DKO mice had increased eosinophils in the spleen after OVA immunization compared with STAT6 KO mice (Fig. 4E). Eosinophils have been reported to produce IL-2 and IL-4, and induce Ag-specific primary and secondary immune responses (31–33). To determine the mechanism of eosinophil recruitment to the spleen, we hypothesized that IP-STAT6 DKO mice had increased production of eosinophil attracting chemokines in the spleen. We found that the levels of CCL2, CCL11, CCL17, and CCL22, but not CXCL12, were significantly increased in the spleen supernatant of IP-STAT6 DKO mice 5 d after OVA/alum sensitization compared with STAT6 KO mice (Fig. 5). Similarly, IP KO mice produced more CCL2, CCL11, CCL17, and CCL22, but not CXCL12, in the spleen compared with WT mice (Fig. 5). As controls, we determined the basal levels of chemokines in the spleen of naive WT, IP KO, STAT6 KO, and IP-STAT6 DKO mice. Naive IP-STAT6 DKO mice had higher levels of CCL2, but similar levels of CCL11 and CCL22, in the spleen compared with naive STAT6 KO mice (Supplemental Fig. 4). Naive IP KO mice had similar levels of CCL2, CCL11, and CCL22 in the spleen as naive WT mice (Supplemental Fig. 4). The levels of CCL17 in the spleen of naive WT, IP KO, STAT6 KO, and IP-STAT6 DKO

FIGURE 5. IP deficiency increased STAT6-independent chemokine production in the spleen during immune sensitization. WT, IP KO, STAT6 KO, and IP-STAT6 DKO mice were injected with OVA/alum i.p. Mouse spleens were harvested at day 5 and the levels of chemokines in the spleen supernatant were determined by ELISA. **(A)** CCL2. **(B)** CCL11. **(C)** CCL17. **(D)** CCL22. **(E)** CXCL12. Data (mean $\pm$ SEM) are combined from three independent experiments. $n = 15$ mice per group. $*p < 0.05$ (one-way ANOVA followed by Bonferroni post hoc tests).



mice were below the limit of detection of ELISA (Supplemental Fig. 4C). These data indicate that IP deficiency increased CCL11, CCL17, CCL22 production to OVA sensitization in both IP-STAT6 DKO and IP KO mice in the spleen, which correlated with augmented infiltration of eosinophils in the spleen (Fig. 4E).

The PGI₂ analogue cicaprost suppressed CD4 T cell proliferation, IL-2 production, and Th2 cytokine expression

IP-STAT6 DKO and IP KO mice had increased OVA-specific IL-5 and IL-13 responses in the spleen compared with STAT6 KO mice and WT mice, respectively (Fig. 4G, 4H), suggesting that endogenous PGI₂ present in the spleen inhibits CD4 T cell activation during OVA sensitization. To test the hypothesis that PGI₂ directly suppresses naive CD4 T cell activation, proliferation, and Th2 cytokine production, we activated WT CD4 T cells with anti-CD3 and anti-CD28 and treated the cells with the PGI₂ analogue cicaprost or vehicle for 3 d. We measured the effect of cicaprost on CD4 T cell proliferation and IL-2 production because IL-2 is a cytokine critical for naive CD4 T cell activation. We found that cicaprost suppressed T cell proliferation (Fig. 6A, 6B) and decreased the total numbers of live cells at day 3 (Fig. 6C). The decreased cell proliferation correlated with attenuated IL-2 production by CD4 T cells (Fig. 6D). Cicaprost also suppressed IL-5, IL-13, and IFN- γ cytokine production and CCL17 and CCL22 chemokine expression by CD4 T cells in a dose-dependent manner compared with vehicle (Fig. 7), suggesting that cicaprost inhibited both Th2 and Th1 differentiation pathways. The levels of CCL2, CCL11, and CXCL12 in the T cell culture supernatant were below the limit of detection. Because Bcl6 and STAT1 have been reported to negatively regulate Th2 inflammatory responses in vivo (34, 35), and STAT6 contributes to Th2 responses (36), we determined the effect of cicaprost on T cell expression of these transcription factors. We found that cicaprost at 100 nM significantly inhibited the mRNA expression of Bcl6, STAT1, and STAT6 by CD4 T cells of WT BALB/c mice (Fig. 7F). Taken together, these results indicate that cicaprost inhibited CD4 T cell activation and Th2 cytokine expression in vitro, and suggest that endogenous PGI₂ in the mouse spleen inhibits CD4 T cell activation and Th2 differentiation.

The PGI₂/IP signaling pathway is critical for indomethacin-mediated proallergic effects

We then tested the hypothesis that IP deficiency abrogates indomethacin-induced, STAT6-independent allergic airway inflammation. We sensitized and challenged WT, IP KO, STAT6 KO, and IP-STAT6

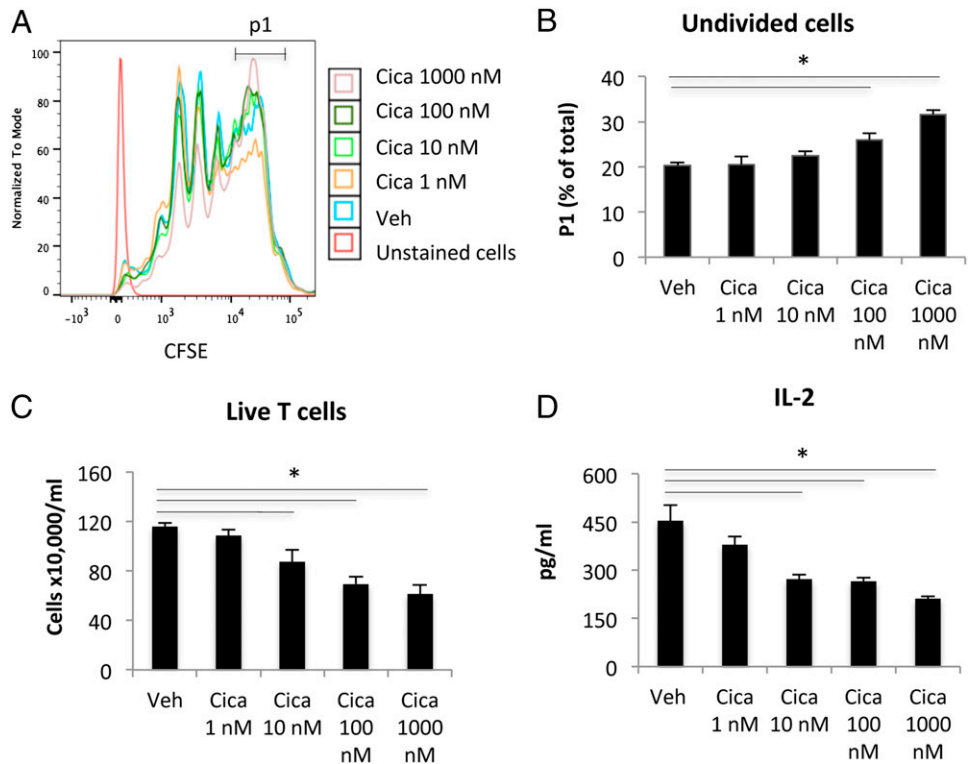
DKO mice with OVA and treated the mice with indomethacin or vehicle during the sensitization phase. We reasoned that if inhibition of endogenous PGI₂ were responsible for indomethacin-augmented allergic inflammation, the proallergic effect of indomethacin would diminish in IP-deficient mice. We found that indomethacin increased IL-5, IL-13, IL-1 α , IL-1 β , and CXCL9 responses in STAT6 KO mice compared with vehicle (Fig. 8A–E). However, indomethacin did not significantly augment the levels of IL-5, IL-13, IL-1 α , IL-1 β , and CXCL9 in the lung of IP-STAT6 DKO mice compared with the vehicle treatment (Fig. 8A–E). We also found that indomethacin increased IL-5 and IL-13 responses in WT mice compared with vehicle (Fig. 8A, 8B). However, indomethacin did not augment the levels of IL-5 and IL-13 in IP KO mice (Fig. 8A, 8B). These results indicate that in the absence of the IP signaling pathway, indomethacin lost its ability to induce further increases in IL-5 and IL-13 responses. Taken together, these data indicate that the PGI₂/IP signaling pathway inhibits indomethacin-induced, STAT6-independent cytokine and chemokine production in this model of allergic airway inflammation.

Because allergen-specific IgE is an indication of effective allergic sensitization, we therefore measured the levels of OVA-specific IgE in the serum samples and found that indomethacin significantly increased OVA-specific IgE levels in OVA-sensitized and -challenged WT mice, but not in IP KO mice, compared with vehicle treatment (Fig. 8F). This result indicates that COX inhibition by indomethacin during immune sensitization augmented allergic sensitization and allergen-specific IgE responses, and IP deficiency diminished the proallergic effect of indomethacin in IP KO mice. We found that STAT6 KO and IP-STAT6 DKO mice did not produce IgE (Fig. 8F).

Discussion

The important regulatory role of COX inhibition in the STAT6-independent allergic inflammatory pathway is indicated by the strong activation of this pathway in indomethacin-treated STAT6 KO mice (3). In this study, we demonstrated that PGI₂ and IP signaling negatively regulated STAT6-independent Th2 responses and allergic inflammation. First, deficiency of IP increased proinflammatory chemokine expression and inflammatory cell infiltration in the lungs of OVA-sensitized and -challenged mice and augmented primary OVA-specific CD4 T cell responses in the spleen in a STAT6-independent manner. Second, the PGI₂ ana-

FIGURE 6. PGI₂ analogue suppressed CD4 T cell activation, proliferation, and IL-2 production. Naive splenic CD4 T cells of WT mice were activated with anti-CD3 and anti-CD28, and treated with cicaprost for 3 d. **(A and B)** Cells were stained with CFSE before the cell culture. **(A)** CFSE intensity of the cells was determined by flow cytometry. **(B)** Cicaprost increased the percentage of less divided cells (p1 in A). **(C)** Live cells were counted at day 3 after trypan blue staining. **(D)** Levels of IL-2 protein in the culture supernatant at day 2. Results are combined data of three independent experiments. Bars represent mean $\pm$ SEM. $n = 9$ –12 wells per treatment group (B–D). $*p < 0.05$ (one-way ANOVA followed by Bonferroni post hoc tests).



logue cicaprost directly suppressed CD4 T cell proliferation and Ag-dependent IL-5 and IL-13 production during naive CD4 T cell activation in vitro. Third, IP deficiency diminished the stimulatory effect of indomethacin treatment during immune sensitization on STAT6-independent allergic Th2 cytokine responses. These data provide the first in vivo evidence that PGI₂ and the IP signal-

ing pathway inhibit STAT6-independent IL-5 and IL-13 responses, inflammatory chemokine responses, and allergic lung inflammation.

Our findings that there are STAT6-independent allergic airway inflammation and Th2 responses are consistent with other studies on alternative STAT6-independent signaling pathways in allergic

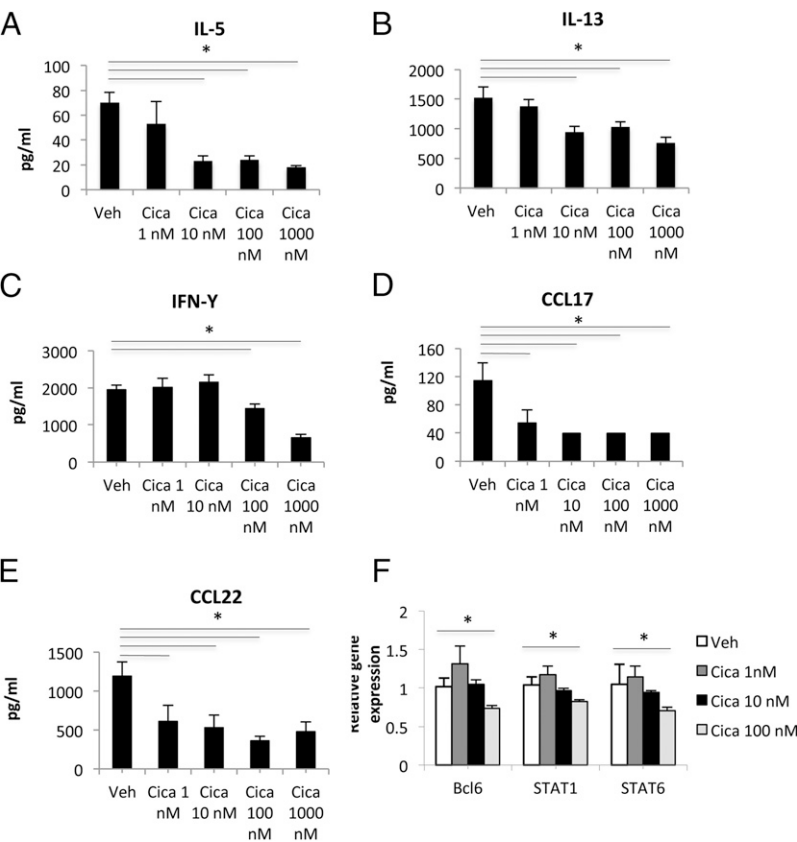


FIGURE 7. PGI₂ analogue suppressed CD4 T cell IL-5, IL-13, IFN- γ , and proinflammatory chemokine production, and inhibited Bcl6, STAT1, and STAT6 expression. Naive splenic CD4 T cells of WT mice were activated with anti-CD3 and anti-CD28, and treated with cicaprost for 3 d. **(A–C)** Levels of IL-5, IL-13, and IFN- γ in the culture supernatant at day 3. **(D and E)** Levels of CCL17 and CCL22 in the culture supernatant at day 3. **(F)** Cultured T cells were harvested at day 2 for RT-PCR to determine the levels of Bcl6, STAT1, and STAT6 mRNA expression (normalized to β -actin expression). Results are combined data of three independent experiments. Bars represent mean $\pm$ SEM. $n =$ total of 9 wells per treatment group. $*p < 0.05$ (one-way ANOVA followed by Bonferroni post hoc tests).

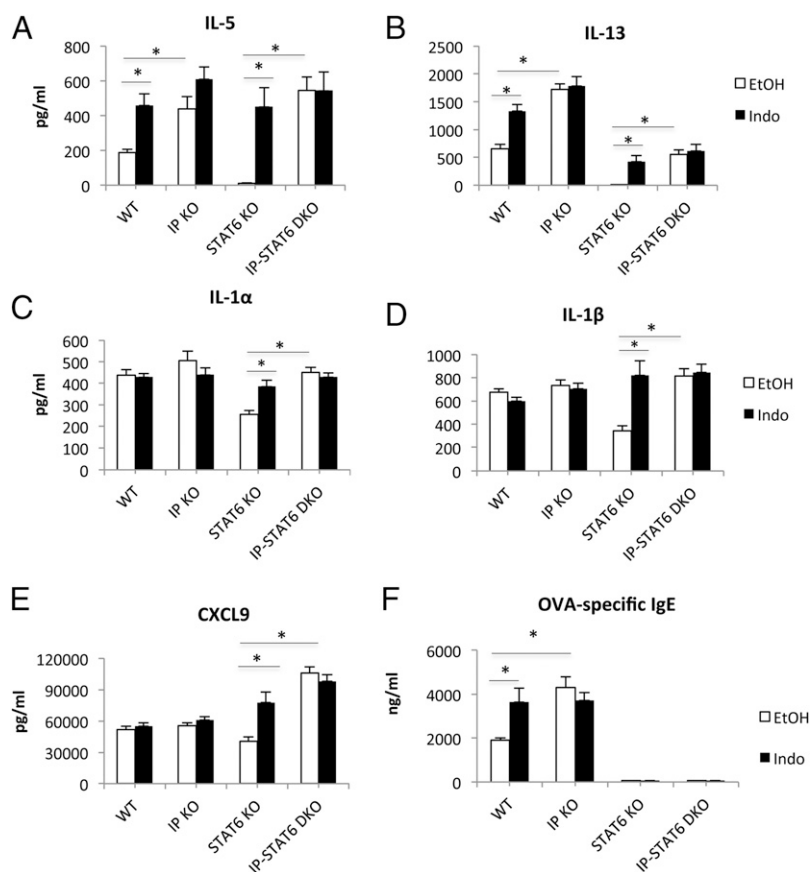


FIGURE 8. IP deficiency attenuated the proallergic effect of COX inhibition. WT, IP KO, STAT6 KO, and IP-STAT6 DKO mice were OVA/alum sensitized i.p. and challenged with OVA aerosols as in Fig. 1A. Mice were treated with either vehicle (ethanol [EtOH]) or indomethacin (Indo) during OVA/alum sensitization phase from day -2 to day 2. Mice were harvested 1 d after the last OVA aerosol. (A and B) Levels of IL-5 and IL-13 in BALF. (C–E) Levels of IL-1 α , IL-1 β , and CXCL9 in the supernatant of the lung homogenate samples. (F) Mouse blood was harvested for determination of OVA-specific IgE in the sera. Results (mean $\pm$ SEM) are combined data of three independent experiments. $n = 14$ – 17 mice per group. * $p < 0.05$ (one-way ANOVA followed by Bonferroni post hoc tests).

inflammation (1, 37). Wang and colleagues (37) reported a STAT6-independent mechanism that partially mediates eosinophil development in the bone marrow and was responsible for the movement of eosinophils into the blood during allergic airway inflammation. They showed that depletion of either IL-5 or CD4 and NK cells, but not CD8 cells, abolished eosinophil development in the bone marrow of STAT6 KO mice, suggesting that IL-5 produced by CD4 and NK cells causes STAT6-independent eosinophil generation in the bone marrow and accumulation in the blood (37). STAT6 KO T cells produced significant amounts of IL-4, IL-5, and IL-13, although less than WT mice, after anti-CD3 stimulation *in vitro*, indicating that STAT6 is not essential for CD4 T cell Th2 cytokine expression (38). Our data suggest that IP deficiency abrogated the suppressive effect of PGI₂ and the IP signaling pathway on CD4 T cell activation, leading to augmented immune sensitization and allergic inflammation in STAT6-deficient mice.

We found that IP-STAT6 DKO mice developed augmented IL-5 and IL-13 cytokine responses and allergic airway inflammation compared with STAT6 KO mice, indicating the STAT6-independent pathway of type 2 immune responses was activated by IP deficiency. However, we observed lower levels of IL-13 production and fewer numbers of eosinophils in BALF after OVA challenge in IP-STAT6 DKO mice than those in IP KO mice. The levels of CCL11, CCL17, and CCL22 in IP-STAT6 DKO mouse lungs after OVA challenge were also lower than those in IP KO mice. Therefore, the STAT6 signaling pathway is important for developing maximal allergic airway inflammation.

In this study, IP deficiency increased the production of CCL11, CCL17, CCL22, CXCL9, and CXCL12 after OVA challenge and augmented infiltration of eosinophils and macrophages in the lung of IP-STAT6 DKO mice compared with STAT6 KO mice. Che-

mokines through specific G protein-coupled receptors expressed by various leukocyte subsets control the migration and function of inflammatory cells into the lung in allergic airway inflammation. CCL11 was originally found to be selective for eosinophils, but recent studies indicate that it was also chemotactic for Th2 cells, basophils, and mast cells (39–41). In humans, administration of CCL11 to the skin induced local eosinophil infiltration in a dose-dependent manner (42). Neutralization of CCL17 and CCL22 by mAbs inhibited early-stage infiltration of Th2 cells to the allergic lung in mice, indicating an important role of these two chemokines in Th2 cell recruitment (43). CXCL12 attracts T cells by binding to CXCR4. A CXCR4 inhibitor has been shown to inhibit OVA-induced allergy inflammation, Th2 responses, and airway hyperresponsiveness in mice, suggesting a stimulatory function of CXCL12–CXCR4 interactions in the pathogenesis of allergic inflammation (44). The upregulation of multiple proinflammatory chemokines including CCL11, CCL17, CCL22, and CXCL12 in IP-STAT6 DKO mice compared with STAT6 KO mice and its association with increased Th2 responses and allergic inflammation suggest that these chemokines promote STAT6-independent allergic responses. In contrast, CXCL9 is an IFN- γ -inducible chemokine and promotes Th1 immune responses and attenuates OVA-induced allergic airway inflammation (22). In this study, the augmentation of STAT6-independent allergic responses in IP-STAT6 DKO mice was associated with increased CXCL9 expression in the lung, suggesting that the antiallergic function of CXCL9 may not be dominant in this research model. However, the increased CXCL9 levels in the lung in OVA-sensitized and -challenged IP-STAT6 DKO mice compared with that in STAT6 KO mice is consistent with the inhibitory effect of IP signaling on epithelial cell release of CXCL9 (28).

We observed basal level expression of CCL2, CCL11, and CCL22 in the spleen (Supplemental Fig. 4) and CCL2, CCL11, CCL17, CCL22, and CXCL12 in the lung (Supplemental Fig. 2) of naive WT, IP KO, STAT6 KO, and IP-STAT6 DKO mice, suggesting a role of these chemokines in tissue homeostasis. Consistent with the finding by Fulkerson and colleagues (35), we found that the expression of CCL11 in OVA-induced allergic airway inflammation in WT mice was STAT6 dependent, because OVA sensitization and challenge did not augment CCL11 production in the lung of STAT6 KO mice compared with that in naive STAT6 KO mice. OVA-challenged IP-STAT6 DKO mice had augmented levels of CCL11 in the lung compared with those in OVA-challenged STAT6 KO mice, indicating that the STAT6-independent expression of this chemokine was activated in the absence of the IP signaling. These data also suggest that CCL11 is only STAT6 dependent when endogenous PGI₂ signaling is present and this is a paradigm shift in the way in which we regard the STAT6 dependency of the expression of this specific chemokine. The augmented chemokine expression in IP-STAT6 DKO mice (CCL11, CCL17, IL-22, CXCL9, and CXCL12) and IP KO mice (IL-11, CCL22, and CXCL12) after OVA challenges compared with STAT6 KO and WT mice, respectively, suggest an inhibitory effect of endogenous PGI₂ on proinflammatory chemokine expression, which is in agreement with the inhibitory effect of PGI₂ on CCL2 and CCL22 production by human monocytes (26, 45).

PGI₂ and the IP signaling pathway may regulate STAT6-independent allergic inflammation not only by promoting chemokine production in the lung during the OVA challenge phase, but also by enhancing primary T cell response in the spleen during the immune sensitization phase. The increased OVA-specific IL-5 and IL-13 responses by IP-STAT6 DKO spleen cells after OVA protein stimulation suggest augmented CD4 T cell activation and Th2 differentiation compared with STAT6 KO mice. The OVA/alum i.p. injection induced stronger inflammation in the spleen of IP-STAT6 DKO mice compared with STAT6 KO mice as evident by greater numbers of total nucleated cells, eosinophils, and neutrophils in the spleen of IP-STAT6 DKO mice. Eosinophils may promote immune sensitization because they can produce cytokines such as IL-2 and IL-4 to enhance T cell activation and Th2 differentiation (31–33). Because cicaprost inhibited CD4 T cell IL-2 expression, cell proliferation (Fig. 6), and IL-5 and IL-13 production in vitro (Fig. 7), IP deficiency may cause increased CD4 T cell activation and Th2 cytokine expression by abrogating the direct suppressive effect of PGI₂ on CD4 T cells in the spleen.

Several studies reported the inhibitory function of PGI₂ in allergic responses (10, 11, 16, 17, 30). Nagao et al. (16) and Takahashi et al. (17) reported that IP-deficient mice had increased Th2 cytokine responses and inflammatory cell infiltration in the lung in OVA-induced allergic airway inflammation, suggesting an antiallergic effect of PGI₂. Jaffar and colleagues (11) reported that CD4 T cells expressed more IL-10 after treatment with a PGI₂ analogue carboprostacyclin and that PGI₂ was responsible for decreased Th2 immune responses and allergic airway inflammation by inhibiting CD4 T cell recruitment to the lung. Other PGs may have a similar effect on allergic inflammation as PGI₂. Kunikata and colleagues (46) reported that PGE₂ inhibited allergic inflammation, Th2 cytokine responses, and airway hyperresponsiveness through EP3. However, the role of PGI₂ and other PGs in STAT6-independent allergic inflammation has not been reported. In this study, the STAT6-independent proallergic effect of indomethacin was attributed mainly to the suppression of the PGI₂/IP signaling pathway because indomethacin-augmented IL-5 and IL-13, IL-1 α , and IL-1 β responses in STAT6 KO mice were not observed in IP-STAT6 DKO mice. Although we focused our

research on PGI₂ regulation of STAT6-independent mechanisms of allergic airway inflammation in this study, we also found that IP KO mice (with functional STAT6) had increased OVA-specific IL5 and IL-13 production, augmented OVA-specific IgE responses, and elevated chemokine (CCL11, CCL22, and CXCL12) production after OVA challenge in the lung compared with WT mice and that splenocytes from IP KO mice had greater Ag-specific IL-5 and IL-13 production compared with splenocytes from WT mice. This suggests that the enhanced immune sensitization and augmented chemokine expression are two mechanisms for increased allergic airway inflammation in IP KO mice reported by Nagao et al. (16) and Takahashi et al. (17).

In summary, allergic airway inflammation is regulated not only by the STAT6-dependent, but also by STAT6-independent pathways. IP deficiency caused activation of the STAT6-independent Th2 cytokine responses and lung inflammation with augmented immune sensitization in the spleen and enhanced proinflammatory chemokine production in the lung. These results, to our knowledge, provide the first evidence that PGI₂/IP signaling suppresses the STAT6-independent pathway of allergic airway inflammation. Furthermore, COX inhibition by indomethacin augmented STAT6-independent type 2 immune responses and allergic airway inflammation mainly by suppressing the PGI₂/IP signaling pathway. PGI₂ and its analogue iloprost are Food and Drug Administration–approved drugs for the clinical use to treat pulmonary hypertension (47). The inhibitory effect of the PGI₂/IP signaling pathway on both STAT6-dependent and -independent allergic inflammation suggest that selective targeting of IP signaling with PGI₂ and its analogue iloprost could be used for treating allergic inflammation and asthma.

Disclosures

The authors have no financial conflicts of interest.

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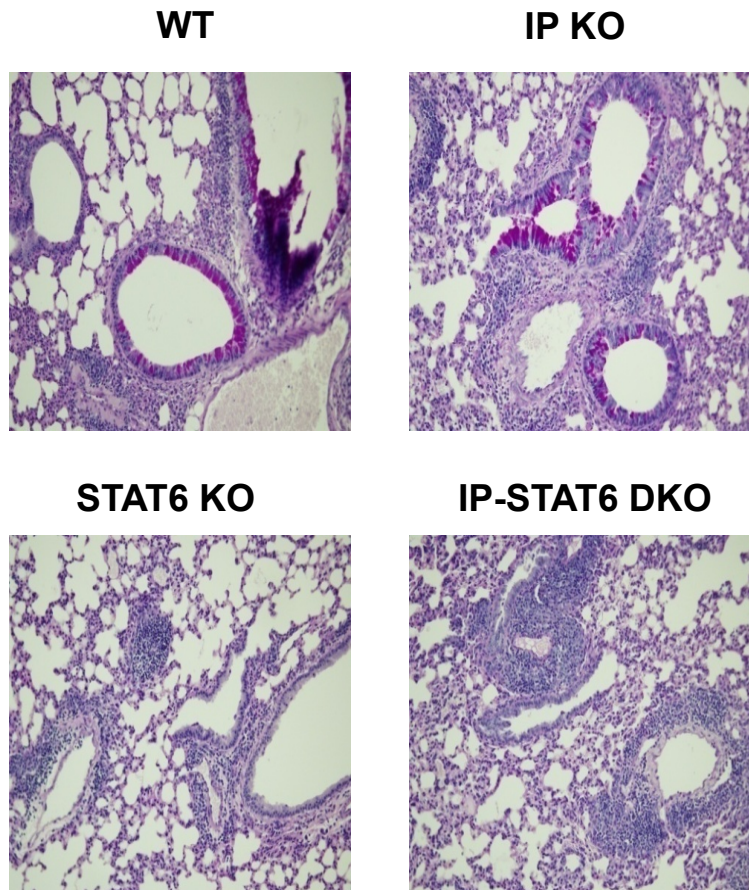


Fig. S1. IP deficiency increased STAT6-independent allergic airway inflammation, but not mucus production in the lung. WT, IP KO, STAT6 KO and IP-STAT6 DKO mice were OVA/alum-sensitized intraperitoneally and challenged with OVA aerosols. The lungs were harvested and the lung sections were PAS stained for pathological analyses.

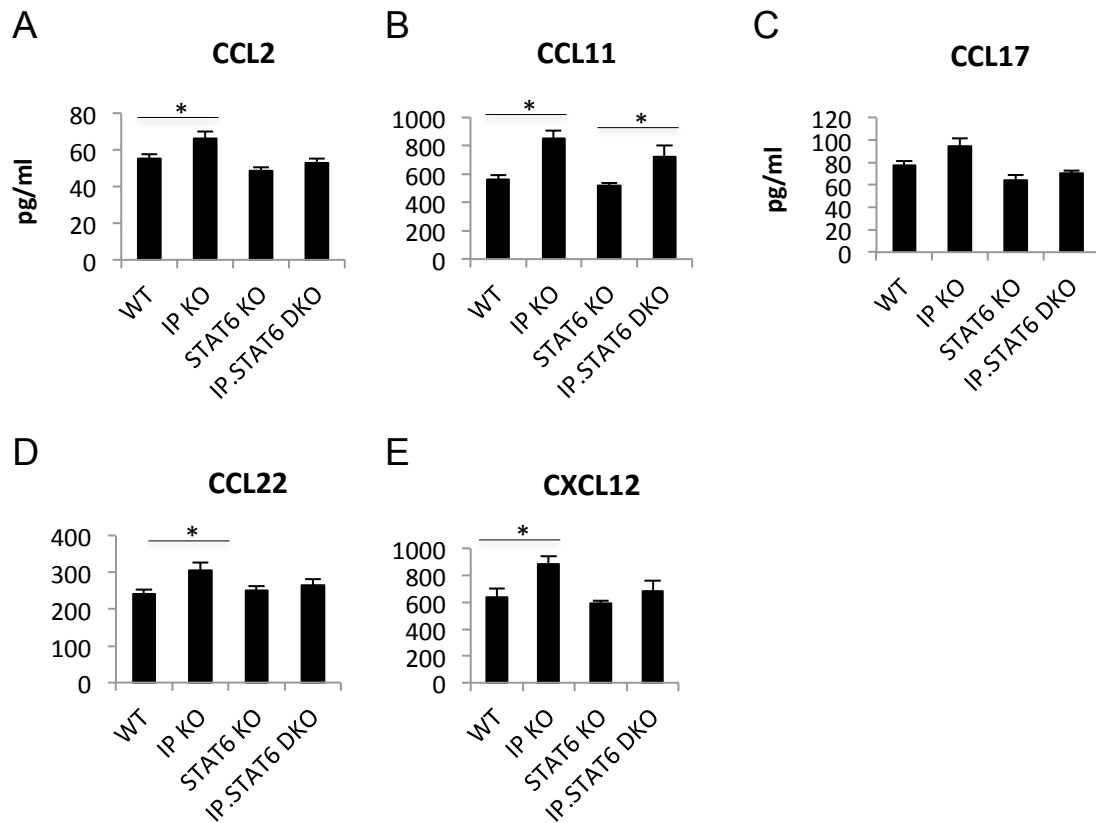


Fig. S2. IP deficiency increased STAT6-independent CCL11 production in the lung of naïve mice. The lungs of naïve WT, IP KO, STAT6 KO and IP-STAT6 DKO mice were harvested and the levels of chemokines were determined by ELISA. (A) CCL2, (B) CCL11, (C) CCL17, (D) CCL22, and (E) CXCL12. Bars represent mean $\pm$ SEM. * $p < 0.05$ (One way ANOVA followed by Bonferroni post hoc tests), $n=5$ mice per group. Data are representative of two independent experiments.

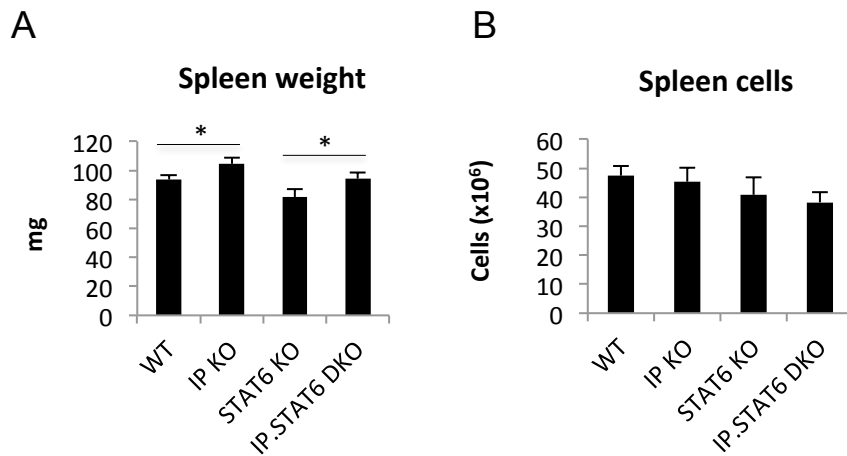


Fig. S3. IP deficiency increased mouse spleen weight, but not total spleen cells in naïve mice. The spleens of naïve WT, IP KO, STAT6 KO and IP-STAT6 DKO mice were harvested and RBC-depleted spleen cells were prepared and stained for differential cell counts (A) Spleen weight. (B) Total spleen cells. Bars represent mean $\pm$ SEM. * $p < 0.05$ (One way ANOVA followed by Bonferroni post hoc tests), $n=5$ mice per group. Data are representative of three independent experiments.

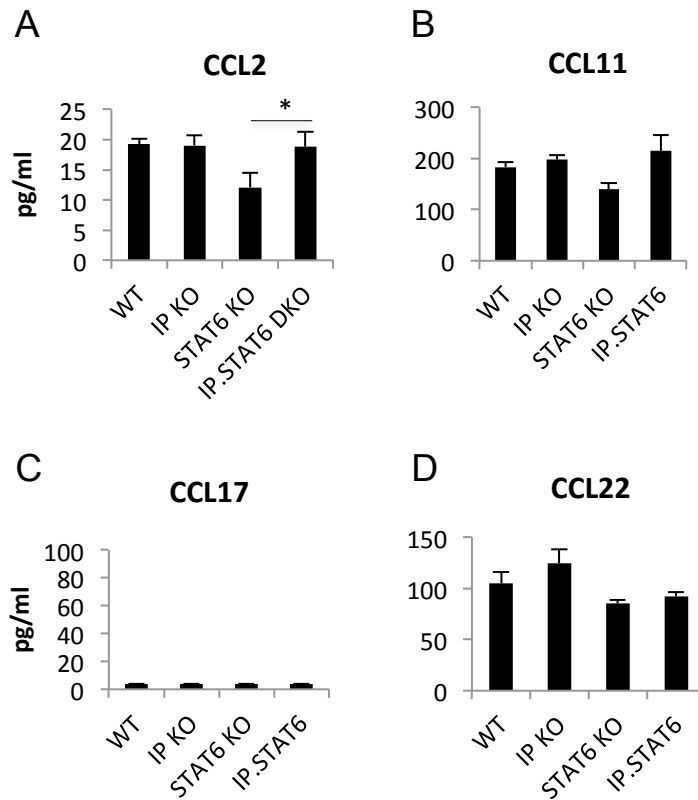


Fig. S4. IP deficiency increased STAT6-independent CCL2 and CXCL12 production in the spleen of naïve mice. The spleens of naïve WT, IP KO, STAT6 KO and IP-STAT6 DKO mice were harvested. The levels of (A) CCL2, (B) CCL11, (C) CCL17, and (D) CCL22 in the spleen supernatant were determined by ELISA. Bars represent mean $\pm$ SEM. * $p < 0.05$ (One way ANOVA followed by Bonferroni post hoc tests), $n=5$ mice per group. Data are representative of three independent experiments.