HHS Public Access

Author manuscript

J Immunol. Author manuscript; available in PMC 2017 September 01.

Published in final edited form as:

J Immunol. 2016 September 1; 197(5): 1577–1586. doi:10.4049/jimmunol.1501063.

PGI_2 suppresses proinflammatory chemokine expression, CD4 T cell activation, and STAT6-independent allergic lung inflammation[1]

Weisong Zhou, Ph.D.[†,2], **Jian Zhang, M.S.**[†], **Kasia Goleniewska, M.S.**[†], **Daniel E. Dulek, M.D.**[†], **Shinji Toki, Ph.D.**[†], **Dawn C. Newcomb, Ph.D.**[†], **Jacqueline Y. Cephus, B.S.**[†], **Robert D. Collins, M.D.**[‡], **Pingsheng Wu, Ph.D.**[†], **Mark R. Boothby, M.D., Ph.D.**[‡], and **R. Stokes Peebles Jr., M.D.**[†,‡]

[†] Division of Allergy, Pulmonary and Critical Care Medicine, Department of Medicine, Vanderbilt University School of Medicine, Nashville, Tennessee, USA

[‡] Department of Pathology, Microbiology and Immunology, Vanderbilt University School of Medicine, Nashville, Tennessee, USA

Abstract

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 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 ovalbumin-induced allergic airway inflammation, we identified that PGI_2/IP receptor signaling was critical for indomethacin-induced STAT6-independent pro-allergic 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 PGI_2 analog 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 PGI_2 and the IP signaling pathway on CD4 T cell activation, inflammatory chemokine production, and allergic sensitization and airway inflammation suggest that PGI_2 and its analog iloprost, both are FDA-approved drugs, may be useful in treating allergic diseases and asthma. In addition, inhibiting PGI_2 signaling by drugs that either block PGI_2 production or restrain IP signaling may augment STAT6-independent pathways of allergic inflammation.

[1]Grant supports: This work was supported by the National Institute of Health (R01 HL 090664, U19 AI 95227, R01 AI 111820, T32 GM 007347, F30 AI118376-01, R56 AI076411), the Department of Veterans Affairs (2I01BX 000624)

[2] Address correspondence to: Weisong Zhou, Ph.D., Division of Allergy, Pulmonary and Critical Care Medicine, Department of Medicine, Vanderbilt University School of Medicine, T-1217 MCN, 21st Ave S, Nashville, TN 37232, USA, Telephone: 615-343-1770; fax: 615-343-7448, weisong.zhou@vanderbilt.edu.

INTRODUCTION

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 pro-allergic effect has not been identified.

The COX pathway of arachidonic acid metabolism is responsible for the formation of PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and thromboxane A_2 (TXA_2), and has immune regulatory 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. PGI_2 functions as an immune modulator through the IP receptor expressed on immune cells such as dendritic cells, T cells and alveolar macrophages (13-15). Multiple studies indicate that PGI_2 suppressed Th2 immune responses and allergic inflammation (10, 11, 13, 14, 16, 17). While the suppressive effect of PGI_2 and its analogs on Th2 immune responses and allergic inflammation is known, the role of PGI_2 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 chemotactic 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 the receptor CCR4 (23). Chemokines are responsible for the cellular composition at inflammatory sites. Studies have shown that the COX pathway and its downstream lipid mediators regulate chemokine expression in inflammation. Inhibition of COX-2 augmented CCL3 (MIP-1α) and CCL5 (RANTES) expression by *Leishmania*-infected peritoneal macrophages (24). PGE_2 attenuated LPS-induced mRNA and protein expression of CXCL-10 (IP-10) and CCL3 in mouse adipose tissue (25). PGI_2 analogs suppressed LPS-induced CCL2 and CCL3 production in human monocytes via the IP receptor and cAMP pathway (26, 27) and inhibited CXCL9 (MIG) and CXCL10 release by human airway epithelial cells (28). However, it is not clear whether PGI_2 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 PGI_2. We hypothesized that PGI_2 and the IP receptor signaling pathway decrease allergen–induced pro-inflammatory chemokine expression and inhibit STAT6-independent allergic airway inflammation. We generated mice

that are deficient in both STAT6 and the IP receptor (IP-STAT6 DKO mice) and used the mice in the ovalbumin (OVA)-induced allergic airway inflammation model. We found that deficiency in the IP signaling pathway increased STAT6-independent pro-inflammatory 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 PGI_2/IP signaling pathway.

MATERIALS AND METHODS

Mice

Wild type (WT) BALB/c mice and STAT6 knockout (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 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.

Induction of allergic airway inflammation

WT, IP KO, STAT6 KO and IP-STAT6 DKO mice were sensitized with an intraperitoneal (i.p.) injection of 100 μl OVA solution containing 10 μg 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 through 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 μg/ml) or vehicle (ethanol) from day -2 (two days prior to OVA-alum injection) to day 2 (2 days 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 μm sections, mounted, and stained with periodic acid-Schiff (PAS) to assess mucus.

Spleen cell culture

Spleens were harvested from either naïve mice or OVA-sensitized mice at day 5 after OVA/alum intraperitoneal injection (Fig. 1A) and ground through 70 μm cell strainers for preparation of single cell suspension. Red blood cells were lysed and the spleen cells were cultured in 96 well flat bottom plates with bovine serum albumin (BSA) (100 μg/ml, as control protein stimulation) or OVA (100 μg/ml) for 3 days. The LPS concentrations in

BSA- or OVA-containing culture media were less than 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 naïve 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 Inc., Herndon, VA) supplemented with 10% FBS (HyClone, Logan, UT), 4 mM L-glutamine, 1 mM sodium pyruvate, 55 μM β-ME, 10 mM HEPES, 100 units/ml penicillin, and 100 μg/ml streptomycin. The cells were stimulated with plate-bound anti-CD3 (5 μg/ml) and anti-CD28 (1 μg/ml) (BD Biosciences, San Diego, CA) in 96 well flat bottom plates and treated with cicaprost at various concentrations or vehicle for 3 days. In T cell proliferation experiments, naïve 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 days 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 Bcl2, 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-1α, IL-1β, IL-6, and 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±standard error of mean (SEM). Statistic 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 pro-allergic 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). Due to the immune suppressive effects of PGI_2 on Th2 immune responses and allergic inflammation (10, 11, 13, 14, 16, 17, 30), we hypothesized that PGI_2 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 hours after the last OVA challenge. We found that IP-STAT6 DKO mice had significantly augmented IL-5, IL-13, IL-1α, and IL-β protein expression, but not IL-4, IL-6, and TNF-α expression, compared to STAT6 KO mice (Fig. 1). IP-STAT6 DKO also had increased numbers of total cells, eosinophils, and macrophages (Fig. 2) in BALF compared to STAT6 KO mice, indicating that IP deficiency activated STAT6-independent allergic 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. 1, B and C) and had increased numbers of total cells, eosinophils, macrophages, and lymphocytes in BALF compared to 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 (Fig. S1), indicating that STAT6 is required for epithelial cell mucus production as reported by others (1).

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

Jaffar and colleagues reported that PGI_2 decreased OVA-induced allergic lung inflammation by inhibiting CD4 T cell recruitment (10). By extension, we hypothesized that the STAT6-independent allergic lung inflammation and Th2 cytokine responses 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 naïve mice. We measured chemokine levels in the lung homogenate of naïve WT, IP KO, STAT6 KO, and IP-STAT6 DKO mice. We found that naïve IP-STAT6 DKO mice had significantly higher levels of CCL11 than naïve STAT6 KO mice (Fig. S2). The levels of CCL2, CCL17, CCL22, and CXCL12 in the lung were similar between naive IP-STAT6 DKO mice and naïve STAT6 KO mice (Fig. S2). In naïve IP KO mice, there were significantly higher levels of CCL2, CCL11, CCL22, and CXCL12 in the lung compared to those of naïve WT mice (Fig. S2). These data suggest that endogenous PGI_2 and the IP signaling inhibit the expression of CCL2, CCL11, CXCL12, and CCL22 in STAT6-sufficient mice, but only suppress 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 hours 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 to 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 inflammation in IP KO mice compared to WT mice (Fig. 2). These data indicate that IP deficiency activated chemokine responses and allergic airway inflammation in a STAT6-independent manner. It should be noted that, compared to 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. 2, A and B), 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 memory responses in OVA-induced allergic airway inflammation (2). We also demonstrated that the pro-allergic 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 days after OVA/alum intraperitoneal 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 to 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 to OVA-sensitized STAT6 KO mice (Fig. 4, C, E, and F)). Similarly, IP KO mice had increased numbers of total nucleated cells and eosinophils in the spleen compared to WT mice (Fig. 4, C and 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 days. We found that OVA, but not BSA, induced IL-5 and IL-13 responses by the spleen cells (Fig. 4, G and H), 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. 4, G and H), 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. 4, G and H). These results suggest that endogenous PGI_2 and the IP signaling suppressed CD4 T cell activation and Th2 differentiation.

To determine whether IP deficiency causes constitutive splenomegaly, we harvested spleens of naïve WT, IP KO, STAT6 KO, and IP-STAT6 DKO mice. We found that the spleen weights of naïve IP-STAT6 DKO mice were 12% greater than those of naïve STAT6 KO (Fig. S3). However, the total numbers of nucleated cells in the spleen were not significantly different between IP-STAT6 DKO and STAT6 KO mice (Fig. S3). Similarly, the spleen weights of naïve IP KO mice were greater (15% more) than those of WT mice, but the total nucleated spleen cell numbers were similar (Fig. S3). 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 to STAT6 KO mice (Fig. 4E). Eosinophils have been reported to produce IL-2, and IL-4, and induce antigen-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 days after OVA/alum sensitization compared to STAT6 KO mice (Fig. 5). Similarly, IP KO mice produced more CCL2, CCL11, CCL17 and CCL22, but not CXCL12, in the spleen compared to WT mice (Fig. 5). As controls, we determined the basal levels of chemokines in the spleen of naïve WT, IP KO, STAT6 KO, and IP-STAT6 DKO mice. Naïve IP-STAT6 DKO mice had higher levels of CCL2, but similar levels of CCL11 and CCL22, in the spleen compared to naïve STAT6 KO mice (Fig. S4). Naïve IP KO mice had similar levels of CCL2, CCL11, and CCL22 in the spleen as naïve WT mice (Fig. S4). The levels of CCL17 in the spleen of naïve WT, IP KO, STAT6 KO, and IP-STAT6 DKO mice were below the limit of detection of ELISA (Fig. S4C). 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 PGI_2 analog 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 to STAT6 KO mice and WT mice, respectively (Fig. 4 G and H), suggesting that endogenous PGI_2 present in the spleen inhibits CD4 T cell activation during OVA sensitization. To test the hypothesis that PGI_2 directly suppresses naïve 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 PGI_2 analog cicaprost or vehicle for 3 days. We measured the effect of cicaprost on CD4 T cell proliferation and IL-2 production as IL-2 is a cytokine critical for naïve CD4 T cell activation. We found that cicaprost suppressed T cell proliferation (Fig. 6, A and B) 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 to 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. Since 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 PGI_2 in the mouse spleen inhibits CD4 T cell activation and Th2 differentiation.

The PGI_2/IP signaling pathway is critical for indomethacin-mediated pro-allergic 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 PGI_2 were responsible for indomethacin-augmented allergic inflammation, the pro-allergic effect of indomethacin would diminish in IP deficient mice. We found that indomethacin increased IL-5, IL-13, IL-1α, IL-1β, and CXCL9 responses in STAT6 KO mice compared to vehicle (Fig. 8, A-E). However, indomethacin did not significantly augment the levels of IL-5, IL-13, IL-1α, IL-1β, and CXCL9 in the lung of IP-STAT6 DKO mice compared to the vehicle treatment (Fig. 8, A-E). We also found that indomethacin increased IL-5 and IL-13 responses in WT mice compared with vehicle (Fig. 8, A and B). However, indomethacin did not augment the levels of IL-5 and IL-13 in IP KO mice (Fig. 8, A and B). 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 PGI_2/IP signaling pathway inhibits indomethacin-induced STAT6-independent cytokine and chemokine production in this model of allergic airway inflammation.

Since 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 to 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 pro-allergic 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 PGI_2 and the IP signaling negatively regulated STAT6-independent Th2 responses and allergic inflammation. First, deficiency of the IP receptor increased pro-inflammatory 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 PGI_2 analog cicaprost directly suppressed CD4 T cell proliferation, and antigen-dependent IL-5, and IL-13 production during naïve 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 PGI_2 and the IP signaling

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

Our findings that there is a STAT6-independent allergic airway inflammation and Th2 responses are consistent with other studies on alternative STAT6-independent signaling pathways in allergic inflammation (1, 37). Wang and colleagues 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 (37). 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, though 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 PGI_2 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 to 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 to STAT6 KO mice. Chemokines 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 monoclonal antibodies 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. An CXCR4 inhibitor has been shown to inhibit OVA-induced allergy inflammation, Th2 responses, and airway hyperresponsiveness in mice, suggesting an stimulatory function of CXCL12-CXCR4 interactions in the pathogenesis of allergic inflammation (44). The upregulation of multiple pro-inflammatory chemokines including CCL11, CCL17, CCL22, and CXCL12 in IP-STAT6 DKO mice compared to STAT6 KO mice and its association with increased Th2 responses and allergic inflammation suggest that these chemokines promotes STAT6-independent allergic responses. On the other hand, 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 anti-allergic 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 to that in STAT6 KO mice is consistent with the inhibitory effect of the IP receptor signaling on epithelial cell release of CXCL9 (28).

We observed basal level expression of CCL2, CCL11 and CCL22 in the spleen (Fig. S4) and CCL2, CCL11, CCL17, CCL22, and CXCL12 in the lung (Fig. S2) of naïve 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 to that in naïve STAT6 KO mice. OVA-challenged IP-STAT6 DKO mice had augmented levels of CCL11 in the lung compared to 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 PGI_2 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 to STAT6 KO and WT mice, respectively, suggest an inhibitory effect of endogenous PGI_2 on pro-inflammatory chemokine expression, which is in agreement with the inhibitory effect of PGI_2 on CCL2 and CCL22 production by human monocytes (26, 45).

PGI_2 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, suggesting augmented CD4 T cell activation and Th2 differentiation compared to STAT6 KO mice. The OVA/alum intraperitoneal injection induced stronger inflammation in the spleen of IP-STAT6 DKO mice compared to 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). Since 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 PGI_2 on CD4 T cells in the spleen.

Several studies reported the inhibitory function of PGI_2 in allergic responses (10, 11, 16, 17, 30). Nagao and Takahashi reported that IP-deficient mice had increased Th2 cytokine responses and inflammatory cell infiltration in the lung in OVA-induced allergic airway inflammation (16, 17), suggesting an anti-allergic effect of PGI_2. Jaffar and colleagues

reported that CD4 T cells expressed more IL-10 after treatment with a PGI_2 analog carboprostacyclin and that PGI_2 was responsible for decreased Th2 immune responses and allergic airway inflammation by inhibiting CD4 T cell recruitment to the lung (11). Other prostaglandins may have similar effect on allergic inflammation as PGI_2. Kunikata and colleagues reported that PGE_2 inhibited allergic inflammation, Th2 cytokine responses, and airway hyperresponsiveness through the EP3 receptor (46). However, the role of PGI_2 and other prostaglandins in STAT6-independent allergic inflammation has not been reported. In this study, the STAT6-independent pro-allergic effect of indomethacin was attributed mainly to the suppression of the PGI_2/IP signaling pathway because indomethacin-augmented IL-5 and IL-13, IL-1α, and IL-1β responses in STAT6 KO mice were not observed in IP-STAT6 DKO mice. While we focused our research on PGI_2 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 to WT mice and that splenocytes from IP KO mice had greater antigen-specific IL-5 and IL-13 production compared to 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 and Takahashi (16, 17).

In summary, allergic airway inflammation is regulated by not only the STAT6-dependent, but also 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 pro-inflammatory chemokine production in the lung. These results provide the first evidence that PGI_2/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 PGI_2/IP signaling pathway. PGI_2 and its analog iloprost are Food and Drug Administration (FDA)-approved drugs for the clinical use to treat pulmonary hypertension (47). The inhibitory effect of the PGI_2/IP signaling pathway on both STAT6-dependent and STAT6-independent allergic inflammation suggest that selective targeting the IP receptor signaling with PGI_2 and its analog iloprost could be used for treating allergic inflammation and asthma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References

1. Kuperman D, Schofield B, Wills-Karp M, Grusby MJ. Signal transducer and activator of transcription factor 6 (Stat6)-deficient mice are protected from antigen-induced airway hyperresponsiveness and mucus production. J.Exp.Med. 1998; 187:939–948. [PubMed: 9500796]
2. Zhou W, Newcomb DC, Moore ML, Goleniewska K, O'Neal JF, Peebles RS Jr. Cyclooxygenase inhibition during allergic sensitization increases STAT6-independent primary and memory Th2 responses. J.Immunol. 2008; 181:5360–5367. [PubMed: 18832692]

3. Hashimoto K, Sheller JR, Morrow JD, Collins RD, Goleniewska K, O'Neal J, Zhou W, Ji S, Mitchell DB, Graham BS, Peebles RS Jr. Cyclooxygenase inhibition augments allergic inflammation through CD4-dependent, STAT6-independent mechanisms. J.Immunol. 2005; 174:525–532. [PubMed: 15611279]
4. Li H, Bradbury JA, Dackor RT, Edin ML, Graves JP, DeGraff LM, Wang PM, Bortner CD, Maruoka S, Lih FB, Cook DN, Tomer KB, Jetten AM, Zeldin DC. Cyclooxygenase-2 regulates Th17 cell differentiation during allergic lung inflammation. Am.J.Respir.Crit Care Med. 2011; 184:37–49. [PubMed: 21474648]
5. Carey MA, Germolec DR, Bradbury JA, Gooch RA, Moorman MP, Flake GP, Langenbach R, Zeldin DC. Accentuated T helper type 2 airway response after allergen challenge in cyclooxygenase-1−/− but not cyclooxygenase-2−/− mice. Am.J.Respir.Crit Care Med. 2003; 167:1509–1515. [PubMed: 12626351]
6. Gavett SH, Madison SL, Chulada PC, Scarborough PE, Qu W, Boyle JE, Tiano HF, Lee CA, Langenbach R, Roggli VL, Zeldin DC. Allergic lung responses are increased in prostaglandin H synthase-deficient mice. J.Clin.Invest. 1999; 104:721–732. [PubMed: 10491407]
7. Zhou W, Goleniewska K, Zhang J, Dulek DE, Toki S, Lotz MT, Newcomb DC, Boswell MG, Polosukhin VV, Milne GL, Wu P, Moore ML, FitzGerald GA, Peebles RS. Cyclooxygenase inhibition abrogates aeroallergen-induced immune tolerance by suppressing prostaglandin I2 receptor signaling. The Journal of allergy and clinical immunology. 2014; 134:698–70500000. [PubMed: 25042746]
8. Peebles RS Jr. Hashimoto K, Sheller JR, Moore ML, Morrow JD, Ji S, Elias JA, Goleniewska K, O'Neal J, Mitchell DB, Graham BS, Zhou W. Allergen-induced airway hyperresponsiveness mediated by cyclooxygenase inhibition is not dependent on 5-lipoxygenase or IL-5, but is IL-13 dependent. J.Immunol. 2005; 175:8253–8259. [PubMed: 16339565]
9. Peebles RS Jr. Hashimoto K, Morrow JD, Dworski R, Collins RD, Hashimoto Y, Christman JW, Kang KH, Jarzecka K, Furlong J, Mitchell DB, Talati M, Graham BS, Sheller JR. Selective cyclooxygenase-1 and -2 inhibitors each increase allergic inflammation and airway hyperresponsiveness in mice. Am.J.Respir.Crit Care Med. 2002; 165:1154–1160. [PubMed: 11956061]
10. Jaffar Z, Ferrini ME, Buford MC, FitzGerald GA, Roberts K. Prostaglandin I2-IP signaling blocks allergic pulmonary inflammation by preventing recruitment of CD4+ Th2 cells into the airways in a mouse model of asthma. J.Immunol. 2007; 179:6193–6203. [PubMed: 17947695]
11. Jaffar Z, Wan KS, Roberts K. A key role for prostaglandin I2 in limiting lung mucosal Th2, but not Th1, responses to inhaled allergen. J.Immunol. 2002; 169:5997–6004. [PubMed: 12421986]
12. Laouini D, Elkhal A, Yalcindag A, Kawamoto S, Oettgen H, Geha RS. COX-2 inhibition enhances the TH2 immune response to epicutaneous sensitization. J.Allergy Clin.Immunol. 2005; 116:390–396. [PubMed: 16083795]
13. Zhou W, Blackwell TS, Goleniewska K, O'Neal JF, FitzGerald GA, Lucitt M, Breyer RM, Peebles RS Jr. Prostaglandin I2 analogs inhibit Th1 and Th2 effector cytokine production by CD4 T cells. J.Leukoc.Biol. 2007; 81:809–817. [PubMed: 17135575]
14. Zhou W, Hashimoto K, Goleniewska K, O'Neal JF, Ji S, Blackwell TS, FitzGerald GA, Egan KM, Geraci MW, Peebles RS Jr. Prostaglandin I2 analogs inhibit proinflammatory cytokine production and T cell stimulatory function of dendritic cells. J.Immunol. 2007; 178:702–710. [PubMed: 17202330]
15. Aronoff DM, Peres CM, Serezani CH, Ballinger MN, Carstens JK, Coleman N, Moore BB, Peebles RS, Faccioli LH, Peters-Golden M. Synthetic prostacyclin analogs differentially regulate macrophage function via distinct analog-receptor binding specificities. J Immunol. 2007; 178:1628–1634. [PubMed: 17237412]
16. Nagao K, Tanaka H, Komai M, Masuda T, Narumiya S, Nagai H. Role of prostaglandin I2 in airway remodeling induced by repeated allergen challenge in mice. Am.J.Respir.Cell Mol.Biol. 2003; 29:314–320. [PubMed: 12676807]
17. Takahashi Y, Tokuoka S, Masuda T, Hirano Y, Nagao M, Tanaka H, Inagaki N, Narumiya S, Nagai H. Augmentation of allergic inflammation in prostanoid IP receptor deficient mice. Br.J.Pharmacol. 2002; 137:315–322. [PubMed: 12237250]

18. Carr MW, Roth SJ, Luther E, Rose SS, Springer TA. Monocyte chemoattractant protein 1 acts as a T-lymphocyte chemoattractant. Proceedings of the National Academy of Sciences of the United States of America. 1994; 91:3652–3656. [PubMed: 8170963]
19. Xu LL, Warren MK, Rose WL, Gong W, Wang JM. Human recombinant monocyte chemotactic protein and other C-C chemokines bind and induce directional migration of dendritic cells in vitro. Journal of leukocyte biology. 1996; 60:365–371. [PubMed: 8830793]
20. Ponath PD, Qin S, Ringler DJ, Clark-Lewis I, Wang J, Kassam N, Smith H, Shi X, Gonzalo JA, Newman W, Gutierrez-Ramos JC, Mackay CR. Cloning of the human eosinophil chemoattractant, eotaxin. Expression, receptor binding, and functional properties suggest a mechanism for the selective recruitment of eosinophils. The Journal of clinical investigation. 1996; 97:604–612. [PubMed: 8609214]
21. Bleul CC, Fuhlbrigge RC, Casasnovas JM, Aiuti A, Springer TA. A highly efficacious lymphocyte chemoattractant, stromal cell-derived factor 1 (SDF-1). J Exp Med. 1996; 184:1101–1109. [PubMed: 9064327]
22. Fulkerson PC, Zimmermann N, Brandt EB, Muntel EE, Doepker MP, Kavanaugh JL, Mishra A, Witte DP, Zhang H, Farber JM, Yang M, Foster PS, Rothenberg ME. Negative regulation of eosinophil recruitment to the lung by the chemokine monokine induced by IFN-gamma (Mig, CXCL9). Proc Natl Acad Sci U S A. 2004; 101:1987–1992. [PubMed: 14769916]
23. Imai T, Nagira M, Takagi S, Kakizaki M, Nishimura M, Wang J, Gray PW, Matsushima K, Yoshie O. Selective recruitment of CCR4-bearing Th2 cells toward antigen-presenting cells by the CC chemokines thymus and activation-regulated chemokine and macrophage-derived chemokine. Int Immunol. 1999; 11:81–88. [PubMed: 10050676]
24. Saha A, Biswas A, Srivastav S, Mukherjee M, Das PK, Ukil A. Prostaglandin E2 negatively regulates the production of inflammatory cytokines/chemokines and IL-17 in visceral leishmaniasis. J Immunol. 2014; 193:2330–2339. [PubMed: 25049356]
25. Tang EH, Cai Y, Wong CK, Rocha VZ, Sukhova GK, Shimizu K, Xuan G, Vanhoutte PM, Libby P, Xu A. Activation of prostaglandin E2-EP4 signaling reduces chemokine production in adipose tissue. Journal of lipid research. 2015; 56:358–368. [PubMed: 25510249]
26. Tsai MK, Hsieh CC, Kuo HF, Lee MS, Huang MY, Kuo CH, Hung CH. Effect of prostaglandin I analogs on monocyte chemoattractant protein-1 in human monocyte and macrophage. Clinical and experimental medicine. 2014
27. Tsai MK, Hsieh CC, Kuo HF, Yang SN, Kuo CH, Huang MY, Tsai YM, Lee MS, Hung CH. Effect of prostaglandin I2 analogs on macrophage inflammatory protein 1alpha in human monocytes via I prostanoid receptor and cyclic adenosine monophosphate. Journal of investigative medicine : the official publication of the American Federation for Clinical Research. 2014; 62:332–339. [PubMed: 24402297]
28. Wilson SM, Sheddan NA, Newton R, Giembycz MA. Evidence for a second receptor for prostacyclin on human airway epithelial cells that mediates inhibition of CXCL9 and CXCL10 release. Molecular pharmacology. 2011; 79:586–595. [PubMed: 21173040]
29. Cheng Y, Austin SC, Rocca B, Koller BH, Coffman TM, Grosser T, Lawson JA, FitzGerald GA. Role of prostacyclin in the cardiovascular response to thromboxane A2. Science. 2002; 296:539–541. [PubMed: 11964481]
30. Idzko M, Hammad H, van NM, Kool M, Vos N, Hoogsteden HC, Lambrecht BN. Inhaled iloprost suppresses the cardinal features of asthma via inhibition of airway dendritic cell function. J.Clin.Invest. 2007; 117:464–472. [PubMed: 17273558]
31. Padigel UM, Lee JJ, Nolan TJ, Schad GA, Abraham D. Eosinophils can function as antigen-presenting cells to induce primary and secondary immune responses to Strongyloides stercoralis. Infection and immunity. 2006; 74:3232–3238. [PubMed: 16714550]
32. Moqbel R, Ying S, Barkans J, Newman TM, Kimmitt P, Wakelin M, Taborda-Barata L, Meng Q, Corrigan CJ, Durham SR, Kay AB. Identification of messenger RNA for IL-4 in human eosinophils with granule localization and release of the translated product. J Immunol. 1995; 155:4939–4947. [PubMed: 7594499]
33. Levi-Schaffer F, Barkans J, Newman TM, Ying S, Wakelin M, Hohenstein R, Barak V, Lacy P, Kay AB, Moqbel R. Identification of interleukin-2 in human peripheral blood eosinophils. Immunology. 1996; 87:155–161. [PubMed: 8666429]

34. Dent AL, Shaffer AL, Yu X, Allman D, Staudt LM. Control of inflammation, cytokine expression, and germinal center formation by BCL-6. Science. 1997; 276:589–592. [PubMed: 9110977]
35. Fulkerson PC, Zimmermann N, Hassman LM, Finkelman FD, Rothenberg ME. Pulmonary chemokine expression is coordinately regulated by STAT1, STAT6, and IFN-gamma. J Immunol. 2004; 173:7565–7574. [PubMed: 15585884]
36. Zhu J, Guo L, Watson CJ, Hu-Li J, Paul WE. Stat6 is necessary and sufficient for IL-4's role in Th2 differentiation and cell expansion. J Immunol. 2001; 166:7276–7281. [PubMed: 11390477]
37. Wang W, Hansbro PM, Foster PS, Yang M. An alternate STAT6-independent pathway promotes eosinophil influx into blood during allergic airway inflammation. PloS one. 2010:6.
38. Takatori H, Nakajima H, Hirose K, Kagami S, Tamachi T, Suto A, Suzuki K, Saito Y, Iwamoto I. Indispensable role of Stat5a in Stat6-independent Th2 cell differentiation and allergic airway inflammation. J.Immunol. 2005; 174:3734–3740. [PubMed: 15749913]
39. Forssmann U, Uguccioni M, Loetscher P, Dahinden CA, Langen H, Thelen M, Baggiolini M. Eotaxin-2, a novel CC chemokine that is selective for the chemokine receptor CCR3, and acts like eotaxin on human eosinophil and basophil leukocytes. J Exp Med. 1997; 185:2171–2176. [PubMed: 9182688]
40. Sallusto F, Mackay CR, Lanzavecchia A. Selective expression of the eotaxin receptor CCR3 by human T helper 2 cells. Science. 1997; 277:2005–2007. [PubMed: 9302298]
41. de Paulis A, Annunziato F, Di Gioia L, Romagnani S, Carfora M, Beltrame C, Marone G, Romagnani P. Expression of the chemokine receptor CCR3 on human mast cells. International archives of allergy and immunology. 2001; 124:146–150. [PubMed: 11306952]
42. Menzies-Gow A, Ying S, Sabroe I, Stubbs VL, Soler D, Williams TJ, Kay AB. Eotaxin (CCL11) and eotaxin-2 (CCL24) induce recruitment of eosinophils, basophils, neutrophils, and macrophages as well as features of early- and late-phase allergic reactions following cutaneous injection in human atopic and nonatopic volunteers. J Immunol. 2002; 169:2712–2718. [PubMed: 12193745]
43. Lloyd CM, Delaney T, Nguyen T, Tian J, Martinez AC, Coyle AJ, Gutierrez-Ramos JC. CC chemokine receptor (CCR)3/eotaxin is followed by CCR4/monocyte-derived chemokine in mediating pulmonary T helper lymphocyte type 2 recruitment after serial antigen challenge in vivo. J Exp Med. 2000; 191:265–274. [PubMed: 10637271]
44. Chen H, Xu X, Teng J, Cheng S, Bunjhoo H, Cao Y, Liu J, Xie J, Wang C, Xu Y, Xiong W. CXCR4 inhibitor attenuates ovalbumin-induced airway inflammation and hyperresponsiveness by inhibiting Th17 and Tc17 cell immune response. Exp Ther Med. 2016; 11:1865–1870. [PubMed: 27168818]
45. Kuo C-HH, Ko Y-CC, Yang S-NN, Chu Y-TT, Wang W-LL, Huang S-KK, Chen H-NN, Wei W-JJ, Jong Y-JJ, Hung C-HH. Effects of PGI2 analogues on Th1- and Th2-related chemokines in monocytes via epigenetic regulation. Journal of molecular medicine (Berlin, Germany). 2010; 89:29–41.
46. Kunikata T, Yamane H, Segi E, Matsuoka T, Sugimoto Y, Tanaka S, Tanaka H, Nagai H, Ichikawa A, Narumiya S. Suppression of allergic inflammation by the prostaglandin E receptor subtype EP3. Nat.Immunol. 2005; 6:524–531. [PubMed: 15806106]
47. Dorris SL, Peebles RS. PGI2 as a regulator of inflammatory diseases. Mediators of inflammation. 2011; 2012:926968. [PubMed: 22851816]

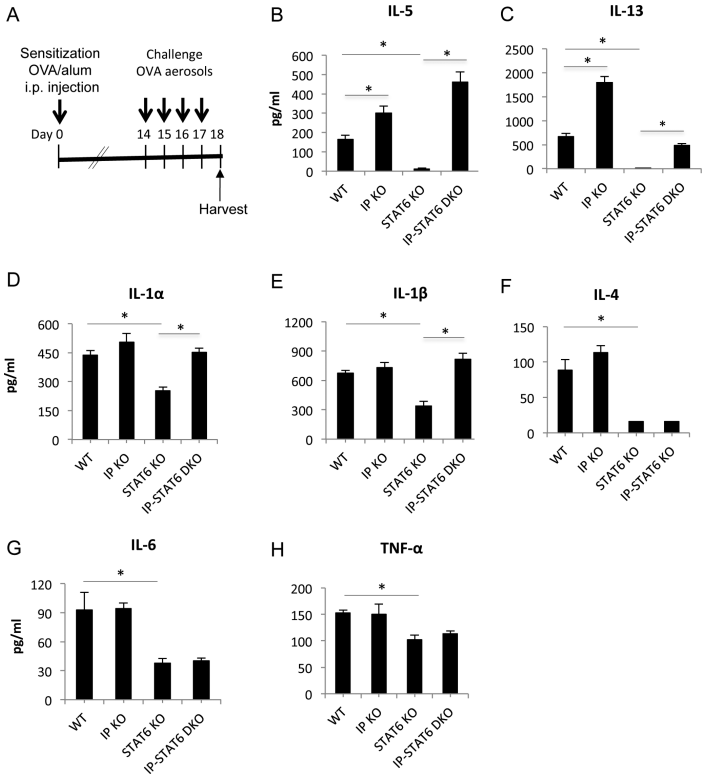

Fig. 1.

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 intraperitoneally and challenged with OVA aerosols. BAL fluid and lungs were harvested one day after the last OVA aerosol for ELISA. (A) The experimental protocol. (B) - (C) IL-5 and IL-13 levels in BAL fluid. (D) – (H) The levels of IL-1α, IL-1β , IL-4, IL-6, and TNF-α in the supernatant of the lung homogenate samples. Data are combined of three independent experiments. Bars represent mean ± SEM. * $p < 0.05$ (One way ANOVA followed by Bonferroni post hoc tests), n=17-20 mice per group.

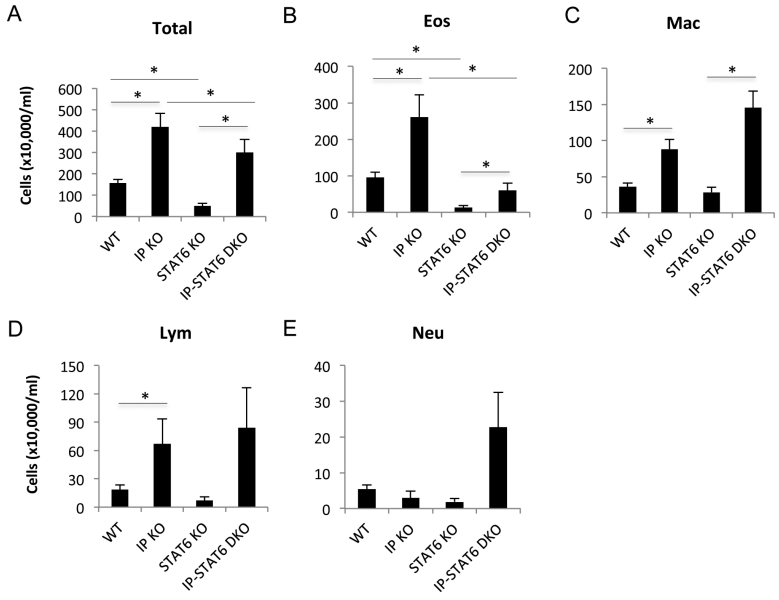

Fig. 2.

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 intraperitoneally and challenged with OVA aerosols. BAL fluid was harvested for total and differential cell counts. (A) Total cells. (B) Eosinophils. (C) Macrophages. (D) Lymphocytes. (E) Neutrophils. Data are combined of three independent experiments. Bars represent mean ± SEM. * $p < 0.05$ (One way ANOVA followed by Bonferroni post hoc tests), n=17-20 mice per group.

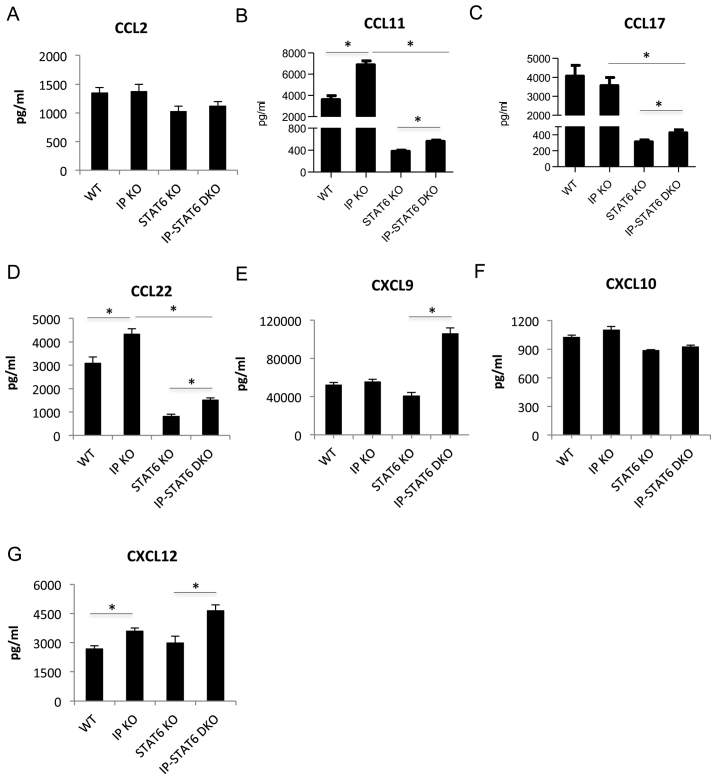

Fig. 3.

IP deficiency increased STAT6-independent pro-inflammatory chemokine production in the lung. WT, IP KO, STAT6 KO and IP-STAT6 DKO mice were OVA/alum-sensitized intraperitoneally 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 of three independent experiments. Bars represent means ± SEM. * $p < 0.05$ (One way ANOVA followed by Bonferroni post hoc tests), n=17-20 mice per group.

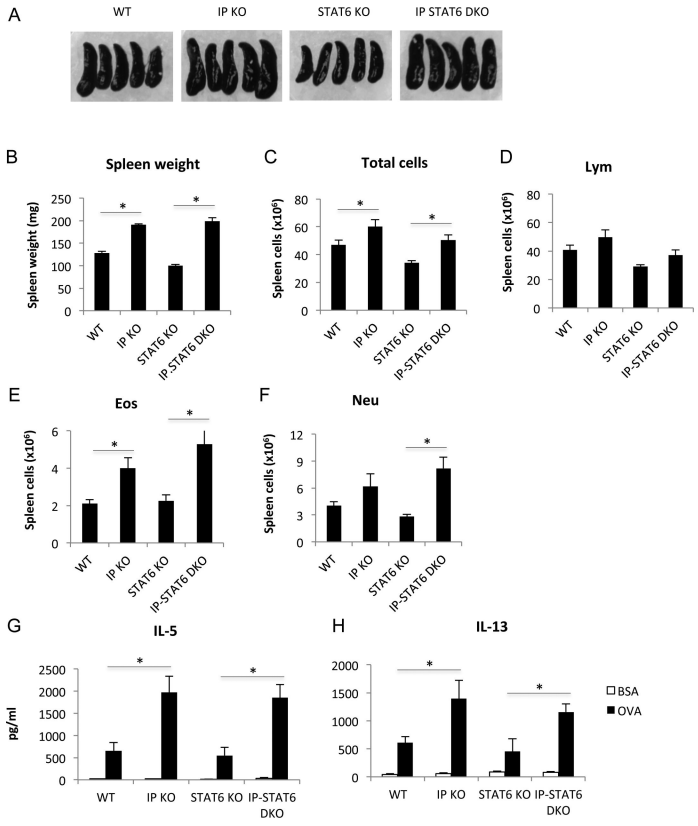

Fig. 4.

IP deficiency increased STAT6-independent primary immune responses to OVA/alum sensitization. Mice were intraperitoneally injected with OVA/alum. Spleens were harvested at 5 days 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)–(H) The spleen cells were cultured with bovine serum albumin (BSA) or OVA protein for 3 days. The levels of IL-5 and IL-13 in the cell culture supernatant were determined by ELISA. Results are combined data of 3 independent experiments. Bars represent means ± SEM in (B) – (H). * $p < 0.05$ (One way ANOVA followed by Bonferroni post hoc tests), n=15 mice per group.

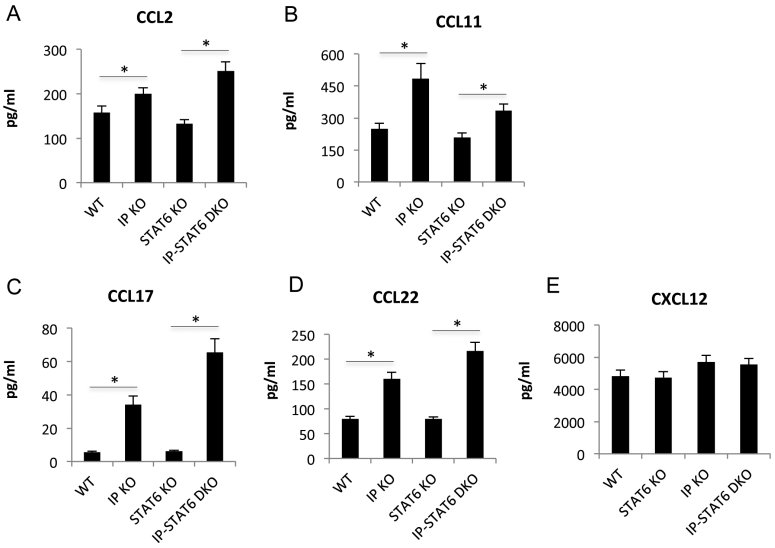

Fig. 5.

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 intraperitoneally. 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 ± SEM) are combined of three independent experiments. * $p < 0.05$ (One way ANOVA followed by Bonferroni post hoc tests), n=15 mice per group.

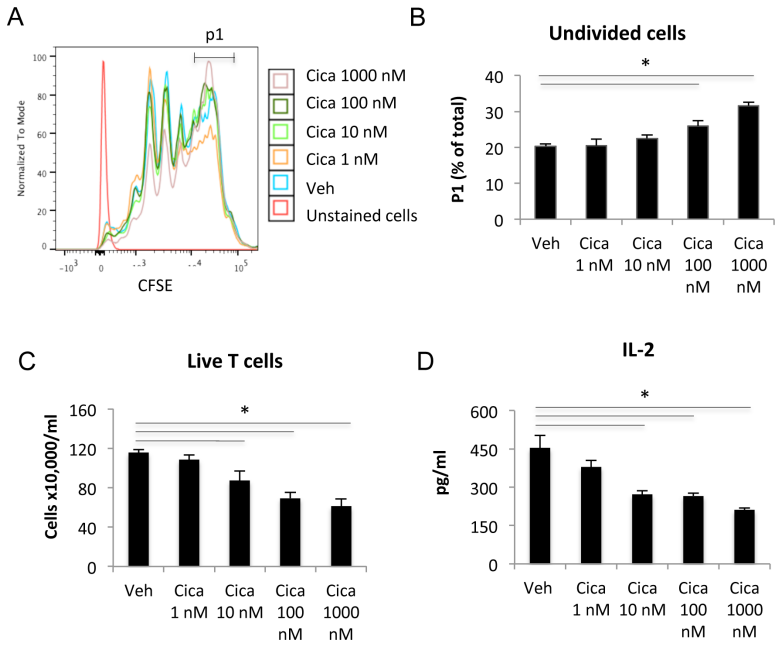

Fig. 6.

PGI_2 analog suppressed CD4 T cell activation, proliferation and IL-2 production. Naïve splenic CD4 T cells of WT mice were activated with anti-CD3 and anti-CD28, and treated with cicaprost for 3 days. (A) – (B) Cells were stained with CFSE prior to the cell culture. (A) CSFE intensity of the cells were 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) The levels of IL-2 protein in the culture supernatant at day 2. Results are combined data of 3 independent experiments. Bars represent means ± SEM. * $p < 0.05$ (One way ANOVA followed by Bonferroni post hoc tests), n=9-12 wells per treatment group (B – D).

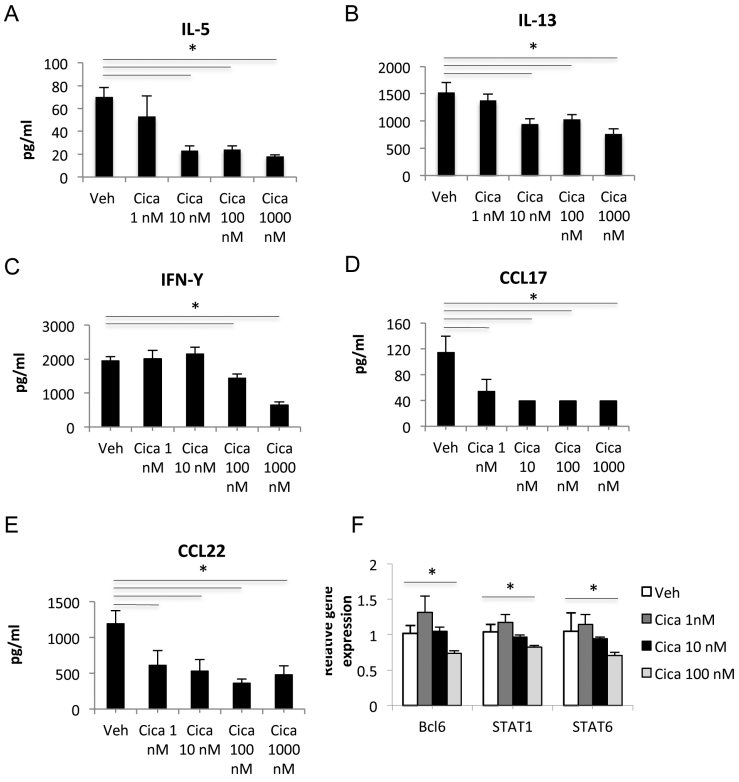

Fig. 7.

PGI_2 analog suppressed CD4 T cell IL-5, IL-13, IFN-γ, and pro-inflammatory chemokine production, and inhibited Bcl6, STAT1, and STAT6 expression. Naïve splenic CD4 T cells of WT mice were activated with anti-CD3 and anti-CD28, and treated with cicaprost for 3 days. (A) - (C) The levels of IL-5, IL-13, and IFN-γ in the culture supernatant at day 3. (D) – (E) The 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 3 independent experiments. Bars represent means ± SEM. * $p < 0.05$ (One way ANOVA followed by Bonferroni post hoc tests), n=total 9 wells per treatment group.

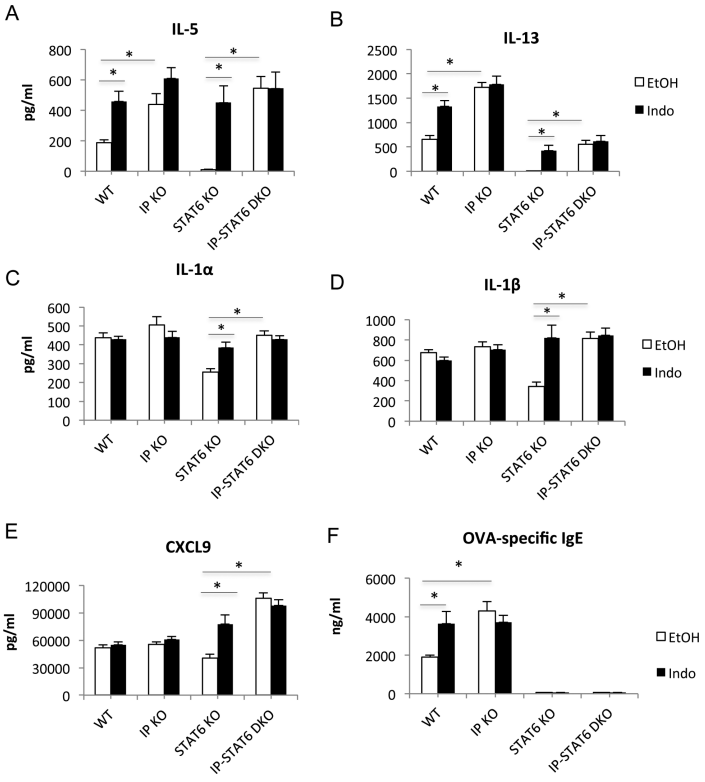

Fig. 8.

IP deficiency attenuated the pro-allergic effect of COX inhibition. WT, IP KO, STAT6 KO and IP-STAT6 DKO mice were OVA/alum sensitized intraperitoneally 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 one day after the last OVA aerosol. (A) - (B) The levels of IL-5 and IL-13 in BAL fluid. (C) - (E) The levels of IL-1α, IL-1β, 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 ± SEM) are combined data of three independent experiments. * $p < 0.05$ (One way ANOVA followed by Bonferroni post hoc tests), n=14-17 mice per group.