

Cyclooxygenase Inhibition Augments Allergic Inflammation through CD4-Dependent, STAT6-Independent Mechanisms¹

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Nonselective cyclooxygenase (COX) inhibition during the development of allergic disease in a murine model causes an increase in type 2 cytokines and lung eosinophilia; however, the mechanisms responsible for this augmented allergen-induced inflammation have not been examined. Ab depletion of CD4 and CD8 cells revealed that the heightened allergic inflammation caused by COX inhibition was CD4, but not CD8, dependent. Allergen sensitization and airway challenge alone led to undetectable levels of IL-5 and IL-13 in the lungs of IL-4, IL-4R α , and STAT6 knockout (KO) mice, but COX inhibition during the development of allergic inflammation resulted in wild-type levels of IL-5 and IL-13 and heightened airway eosinophilia in each of the three KO mice. These results indicate that the effect of COX inhibition was independent of signaling through IL-4, IL-4R α , and STAT6. However, whereas COX inhibition increased IgE levels in allergic wild-type mice, IgE levels were undetectable in IL-4, IL-4R α , and STAT6 KO mice, suggesting that IL-13 alone is not a switch factor for IgE synthesis in this model. These results illustrate the central role played by products derived from the COX pathway in the regulation of allergic immune responses. *The Journal of Immunology*, 2005, 174: 525–532.

Allergic inflammation and allergen-induced airway hyper-responsiveness are augmented when mice are treated with either a nonselective cyclooxygenase (COX)⁴ inhibitor or selective COX-1 or COX-2 inhibitors during allergic sensitization (1, 2). This amplified allergic response resulting from COX inhibition is characterized by an increase in lung IL-5 and IL-13, as well as lung interstitial eosinophilia (1). However, the cells and signaling mechanisms by which COX exerts its immunoregulatory effect in the development of allergic inflammation have not been defined. Key cells involved in the development of the allergic response include APCs such as dendritic cells and macrophages, T lymphocytes, B lymphocytes, and mast cells (3). However, T lymphocytes and their type 2 products, including IL-4, IL-5, IL-9, and IL-13, are believed to be essential regulators of allergic disease (4).

IL-4 is a critical factor for the development of type 2 immune responses (5). When IL-4 binds to its receptor, an IL-4 receptor complex is formed which, in hemopoietic cells, is composed of

IL-4R α and the common γ -chain of the receptors for IL-2, IL-4, IL-7, IL-9, and IL-15 (reviewed in Ref. 6). IL-4R α is also a functional component of the IL-13 receptor complex and is the subunit responsible for intracellular signal transduction after stimulation by IL-13. Disruption of IL-4R α impairs type 2 cytokine production. The binding of IL-4 to its receptor induces the transphosphorylation of Janus kinase (JAK)1 and JAK3, activating these kinases and initiating the early events of signal transduction. IL-4 phosphorylation of JAK1 and JAK3 leads to phosphorylation of IL-4R α and phosphorylation of STAT6. The phosphorylated STAT6 forms a dimer that allows it to enter the nucleus and bind to specific DNA sequences in IL-4-responsive genes. Therefore, IL-4, IL-4R α , and STAT6 are critical elements in the development of type 2 immune responses and allergic disease (6).

We therefore hypothesized that the allergic inflammation augmented by COX inhibition is T cell dependent and also dependent on signaling through the IL-4R α and STAT6 pathways. To test this hypothesis, we used a well-characterized model of allergic sensitization, using OVA as an Ag in wild-type (WT), IL-4 knockout (KO), IL-4R α KO, and STAT6 KO BALB/c mice. To inhibit COX activity and PG synthesis, we treated the mice during the allergen sensitization protocol with indomethacin, a nonselective COX inhibitor. The dose of indomethacin used inhibits PGE₂ production and does not cause illness in experimental animals (7).

Materials and Methods

Mice

Pathogen-free 8-wk-old female IL-4 KO, IL-4R α KO, and STAT6 KO BALB/c mice were purchased from The Jackson Laboratory. In caring for animals, the investigators adhered to the Guide for the Care and Use of Laboratory Animals prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (revised 1996).

Allergen sensitization and challenge protocol

OVA-sensitized and challenged mice were injected i.p. with 0.1 ml (10 μ g) of OVA (chicken OVA, grade V; Sigma-Aldrich) complexed with 20 mg of Al(OH)₃ on day 0. On days 14 and 15, these mice were placed in an

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⁴ Abbreviations used in this paper: COX, cyclooxygenase; BAL, bronchoalveolar lavage; JAK, Janus kinase; KO, knockout; WT, wild type; NSAID, nonsteroidal antiinflammatory drug; RPA, RNase protection assay; MCP-1, monocyte chemoattractant protein-1; MIP-1 β , macrophage-inhibitory protein-1 β .

acrylic box and exposed to aerosols of 1% OVA diluted in sterile PBS using an ultrasonic nebulizer (Ultraneb 99; DeVilbiss) for 40 min each day. Mock-sensitized mice were injected with 20 mg of $\text{Al}(\text{OH})_3$ on day 0 and did not undergo aerosol exposure.

COX inhibitor administration

Indomethacin (30 $\mu\text{g}/\text{ml}$) was administered in the drinking water starting on day -2 (2 days before the i.p. injection of OVA complexed with $\text{Al}(\text{OH})_3$). An indomethacin stock was made by dissolving 150 mg of indomethacin in 50 ml of ethanol. Three times per week throughout the experimental protocol, 2 ml of the indomethacin stock solution were added to 200 ml of water in the water bottles of the animals. The water of the control mice was also changed three times per week, and 2 ml of ethanol were added to 200 ml of water in the water bottles of those mice. OVA-sensitized and challenged mice treated with vehicle in the drinking water are designated OVA, whereas mock-sensitized mice treated with vehicle in drinking water are designated mock. OVA-sensitized and challenged mice treated with indomethacin in drinking water are designated OVA-indomethacin, whereas mock-sensitized mice treated with indomethacin in drinking water are designated mock-indomethacin. To confirm COX inhibition in the indomethacin-treated mice, we measured both serum thromboxane and bronchoalveolar lavage (BAL) fluid PGE_2 levels from all groups of OVA and OVA-indomethacin mice. Serum thromboxane was measured after the serum was incubated with ionophore to stimulate maximal platelet thromboxane production. Those mice that were treated with vehicle in the drinking water did not have any inhibition of plasma thromboxane generation, whereas those mice treated with indomethacin had significantly inhibited thromboxane generation (data not shown) (2). PGE_2 in BAL fluid was measured by a modified stable isotope dilution assay that used gas chromatography-negative ion chemical ionization-mass spectrometry as previously described (1). PGE_2 was detected in BAL fluid only in the WT OVA group.

In vivo T cell depletion

CD4 cells were depleted by treatment with GK1.5 (anti-CD4) Abs, and CD8 cells were depleted with 2.43 (anti-CD8) Abs, respectively; whereas the isotype-matched HB151 Ab was used as a control. Each mouse was injected with either 200 μg of GK1.5 Ab, 200 μg of 2.43 Ab, or 200 μg of HB151 Ab i.p. on day 12 (2 days before the first OVA aerosol exposure), day 13, and day 14. The efficacy of these treatments in affecting CD4 and CD8 cell subsets had been previously determined by FACS of heparinized whole peripheral blood using FITC- or PE-conjugated rat anti-mouse Ab to CD4 or CD8, respectively (BD Pharmingen) with >98% of CD4 or CD8 cells depleted 21 days after the last Ab treatment. Each Ab conjugate was incubated with 200 μl of whole blood for 30 min at room temperature. The RBC were lysed with a buffered ammonium chloride solution, and the remaining lymphocytes were washed twice and then resuspended in PBS containing 5% FBS before analysis on an EPIC 753 FACS (Coulter).

Quantitation of IL-5 and IL-13 in lung tissues

Levels of IL-5 and IL-13 in lung tissues of mice were measured using commercially available ELISA kits (R&D Systems) according to the manufacturer's protocols. On day 16, the lungs from four mice in each group were analyzed for cytokine levels using ground lung supernatants as previously described (8).

Cytokine, chemokine, and chemokine receptor detection by RNase protection assay (RPA)

Probes for a panel of cytokines, chemokines, and chemokine receptors (all from BD Pharmingen) were used according to the manufacturer's instructions as previously described (1).

Quantitation of chemokine protein by laser-based fluorescent analytical testing

Levels of RANTES and monocyte chemoattractant protein-1 (MCP-1) in lung tissues of mice were measured with a commercially available LIN-COPlex mouse cytokine/chemokine kit (Linco Research) using fluorescently labeled microsphere beads and a Luminex reader (Luminex).

Bronchoalveolar lavage

The animals were given a lethal injection of pentobarbital. BAL was then performed by instilling 800 μl of normal saline through the tracheostomy

tube and then withdrawing the fluid with gentle suction via the syringe. The typical BAL fluid return was 500–600 μl . White blood cells were counted on a hemocytometer. Cytologic examination was performed on cytospin preparations (Shandon Southern Instruments). Cytospin slides were fixed and stained using Diff Quik (American Scientific Products). Differential counts were based on counts of 100 cells using standard morphological criteria to classify the cells as either eosinophils, lymphocytes, or other mononuclear leukocytes (alveolar macrophages and monocytes).

Protocol for examining lung sections

The mice were sacrificed by cervical dislocation on day 16, and the lung block was removed. The lung tissue was stored in 4% paraformaldehyde, paraffin-embedded, cut in 6- μm sections, mounted, and stained with H&E for routine histology, periodic acid-Schiff to assess mucus, and Wright-Giemsa stain to evaluate eosinophils. Slides were examined by one observer in a blinded manner as previously described (8, 9).

Analysis of intracellular cytokines by flow cytometry

To obtain sufficient lymphocytes for analysis, the lymphocytes from two mice harvested on day 16 were pooled to provide a single data point. Whole lung lymphocytes were isolated by Ficoll-Hypaque (Sigma-Aldrich; 1.09 specific gravity) cushion centrifugation. The lymphocytes were cultured at 37°C in Iscove's medium supplemented with 10% FCS (HyClone), 0.01 mM nonessential amino acid mix, 2 mM sodium glutamate, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 5.5 μM 2-ME (all from Invitrogen Life Technologies) for 6 h in the presence of GolgiStop (BD Pharmingen) with 10 ng/ml PMA (Sigma-Aldrich) and 1 μM ionomycin (Sigma-Aldrich). CD4 cells were marked using PE-anti-CD4 (BD Pharmingen). Cells were fixed for 20 min in Cytofix/Cytoperm (BD Pharmingen) and then stained with FITC-anti-IL5 (BD Pharmingen). Samples were analyzed using a FACSCalibur BD Biosciences flow cytometer. The forward scatter and side scatter properties of the cells were used to exclude dead cells from analysis. Between 10,000 and 30,000 cells were analyzed.

Total IgE concentrations

Before sacrifice on day 16, sera were collected from sensitized mice and then analyzed by ELISA to determine levels of total IgE. To determine total IgE levels, 96-well Immunolon II plates (Nunc) were coated with a 1/200 dilution of rat monoclonal anti-murine IgE clone LO-ME-3 (Serotec). Plates were washed with PBS-0.5% Tween 20 and blocked with PBS-1% BSA for 1 h. The plates were then washed before adding 100 μl of serum diluted 1/300 in PBS for WT mice and 1/10 in PBS for the IL-4 KO, IL-4R α KO, and STAT6 KO mice. Plates were incubated overnight and washed, and 100 μl of rat anti-mouse IgE clone LO-ME-2 (Serotec) diluted 1/2000 were added to each well. After 1 h of incubation at 37°C, the plates were again washed, and HRP activity was determined with a tetramethylbenzidine (Sigma-Aldrich) developing solution (1% tetramethylbenzidine in DMSO, 0.001 M sodium acetate, and 0.45% H_2O_2 final concentration). Substrate development was stopped with 2.5 M H_2SO_4 and OD was measured at 450 nm. Concentration was extrapolated by use of an IgE standard (Maine Biotech). The lower limit of detection for the assay was 32.5 ng/ml.

Statistical analysis

Results are expressed as mean $\pm$ SEM. Levels of cytokines that were below the limit of detection for the assay were assigned a value one-half that of the lower limit of detection (31.25 pg/ml for IL-5 and 62.5 pg/ml for IL-13) for statistical analysis. Measurements of cytokines by ELISA, chemokines by RPA, cells by flow cytometry, cell counts and differentials, and IgE by ELISA were analyzed by unpaired *t* test. Differences were considered to be significant if $p < 0.05$.

Results

The augmented IL-5 and IL-13 production resulting from COX inhibition during the development of allergic inflammation is dependent on CD4⁺ cells

Previously, we found that levels of IL-5 in ground lung supernatants peaked on day 16 of our protocol (2); therefore, we measured cytokines on this day (2). In this experiment, we first examined cytokine production in six groups of WT mice. OVA-indomethacin and OVA groups were separately treated with either anti-CD4

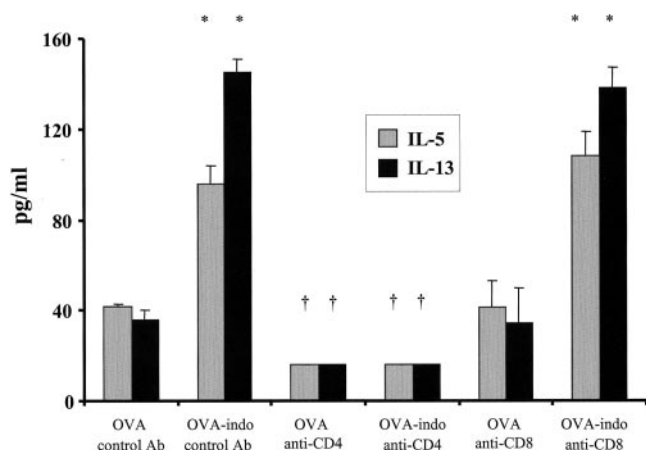


FIGURE 1. Concentrations of IL-5 and IL-13 on day 16 in the lung supernatants from WT mice ($n = 3-5$ for each group). The mice were treated with anti-CD4, anti-CD8, or an irrelevant Ab. Data are representative of two separate experiments. *, $p < 0.05$ compared with the OVA-control Ab and OVA-anti CD8 antibody group. †, $p < 0.05$ compared with the groups treated with either the control antibody or the anti-CD8 Ab. Indo = indomethacin.

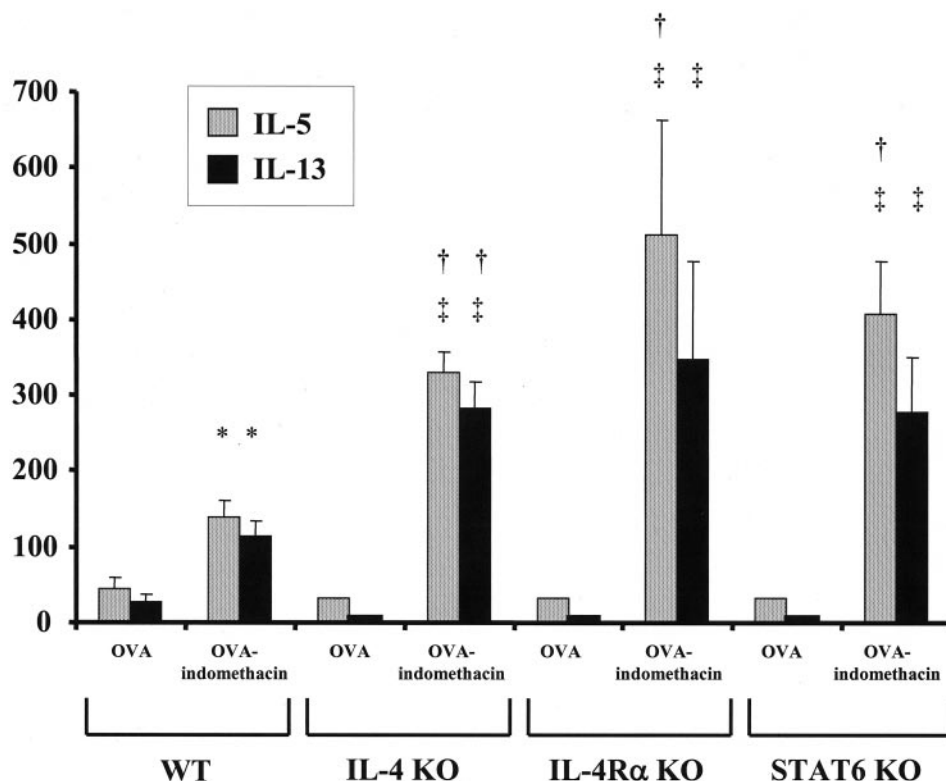
Ab, anti-CD8 Ab, or an isotype-matched control Ab. Of the two groups that were injected with the isotype-matched control Ab, the OVA-indomethacin mice had significantly increased levels of IL-5 and IL-13 in ground lung supernatants compared with the mice treated with vehicle (Fig. 1). There was also a significant increase in the lung levels of IL-5 and IL-13 in the OVA-indomethacin mice compared with the OVA mice in the anti-CD8-treated groups; however, there was no difference in these two CD8-depleted groups compared with their respective groups treated with

the isotype-matched Ab, revealing that the augmented allergic phenotype witnessed with COX inhibition was not CD8 dependent. However, CD4 depletion led to undetectable lung levels of IL-5 and IL-13 in both the OVA-indomethacin and OVA mice. There were undetectable lung levels of IL-5 and IL-13 in both the indomethacin- and vehicle-treated nonsensitized mice (data not shown). Therefore, the augmented type 2 cytokine production resulting from COX inhibition during the development of allergic inflammation is T lymphocyte dependent.

IL-5 and IL-13 are increased to WT levels in OVA-indomethacin IL-4 KO, IL-4R α KO, and STAT6 KO mice

Given that the increased type 2 cytokine production caused by COX inhibition during the development of allergic inflammation was T cell dependent, we wished to determine the signaling stage of type 2 development when COX inhibition exerted its proinflammatory activity. Cytokines were measured in ground lung supernatants of allergically sensitized and challenged mice on day 16. Levels of IL-5 and IL-13 were significantly greater in ground lung supernatants of OVA-indomethacin mice than were the levels seen in the OVA mice on day 16 ($p < 0.05$; Fig. 2). In fact, the OVA-indomethacin IL-4 KO, IL-4R α KO, and STAT6 KO mice had levels of IL-5 and IL-13 that were comparable with or greater than the levels found in WT mice. IFN- γ was undetectable in the lung supernatants from all of the groups. RPAs were performed on the lungs from mice harvested on day 16 (Fig. 3). There was no detectable mRNA for IL-5 or IL-13 in the OVA IL-4 KO, IL-4R α KO, or STAT6 KO groups; however, mRNA for IL-5 and IL-13 in the OVA-indomethacin IL-4 KO, IL-4R α KO, or STAT6 KO groups was present at the levels seen in the WT mice. There was no detectable mRNA for IFN- γ in any of the groups. Therefore, COX inhibition increased the type 2 cytokines IL-5 and IL-13 independent of IL-4, IL-4R α , and STAT6 signaling.

FIGURE 2. Concentrations of IL-5 and IL-13 in the lung supernatants from WT, IL-4 KO, IL-4R α KO, and STAT6 KO mice on day 16 ($n = 4-5$ for each group on each day). Data are representative of three separate experiments. *, $p < 0.05$ compared with the OVA group. †, $p < 0.05$ compared with the WT OVA and OVA-indomethacin group. ‡, $p < 0.01$ compared with the IL-4 KO, IL-4R α KO, and STAT6 KO OVA groups.



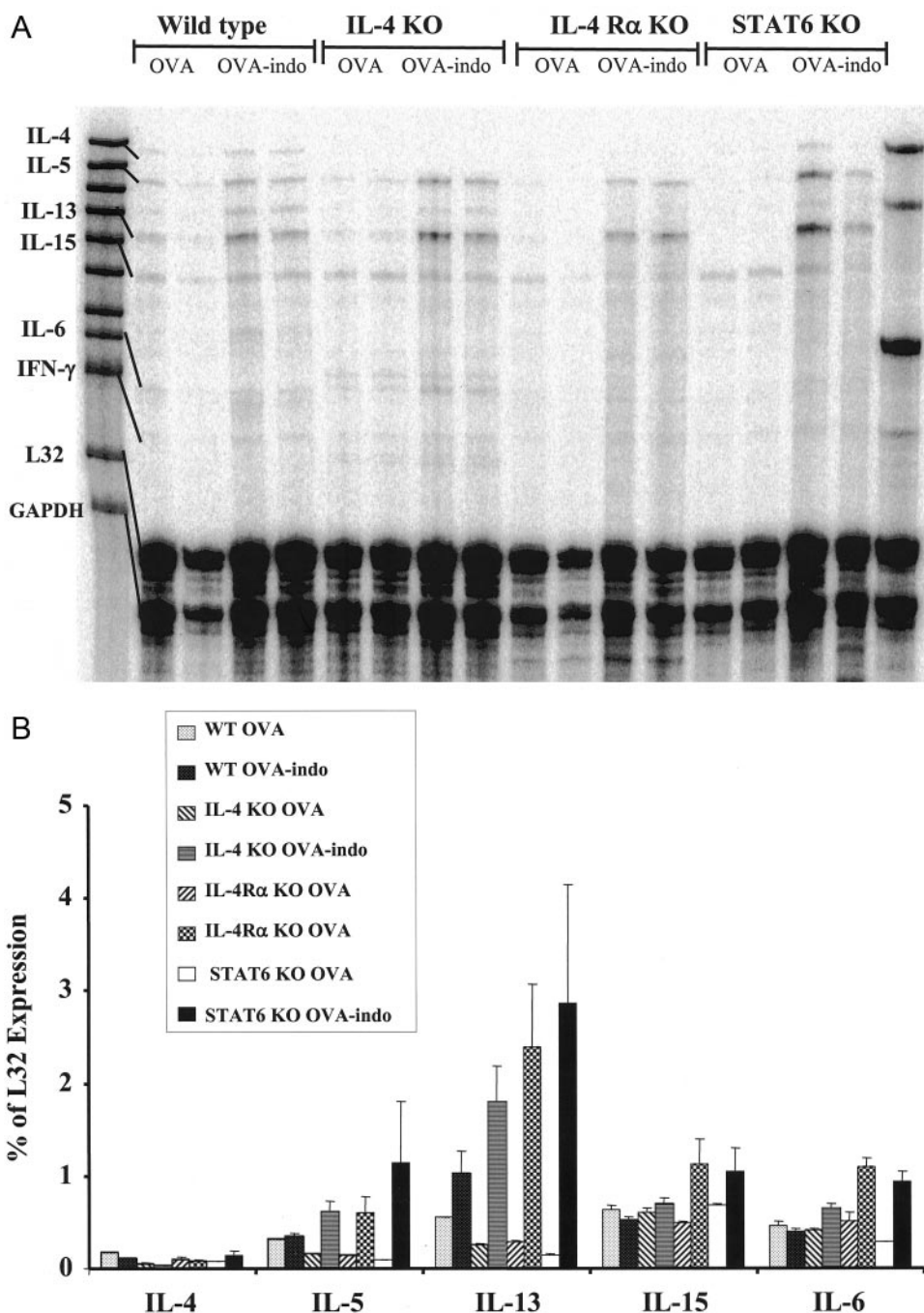


FIGURE 3. A, RPA showing cytokine mRNA present in lungs from WT, IL-4 KO, IL-4Rα KO, and STAT6 KO harvested on day 16. L32, ribosomal mRNA used as a loading control. B, Data from an RPA showing cytokine mRNA present in lungs harvested on day 16. Data are expressed as mRNA for each cytokine as a percentage of L32. indo, Indomethacin.

COX inhibition caused differential regulation of chemokine mRNA expression in the lung

RPAs were performed on the lungs from mice harvested on day 16 (Fig. 4, A and B). There were no detectable differences in chemokine expression between the WT OVA-indomethacin and OVA groups. In the IL-4 KO mice, there was no difference in mRNA levels of lymphotactin, RANTES, or macrophage-inhibitory protein-1β (MIP-1β) between the OVA-indomethacin and OVA groups. However, the lung mRNA expression of eotaxin, MIP-1α, and MCP-1 was greater in the IL-4 KO OVA-indomethacin group than in the OVA group. The STAT6 KO OVA-indomethacin mice had greater lung expression of MCP-1 than did the STAT6 KO OVA group. There was undetectable expression of all the other chemokines, including eotaxin, in the IL-4Rα KO and STAT6 KO OVA-indomethacin and OVA groups. We confirmed the RPA

results independently for selected chemokines by measuring protein levels of RANTES and MCP-1 by using fluorescently labeled microsphere beads. Therefore, the increase in COX inhibition-mediated chemokine expression, with the exception of MCP-1, is dependent on signaling through IL-4Rα and STAT6, suggesting that these chemokines may be primarily induced by IL-13 rather than by IL-4.

COX inhibition increased allergen-induced cellularity of BAL fluid in STAT6 KO mice

Because signaling through IL-4, IL-4Rα, and STAT6 is a critical factor in the development of allergic inflammation, we measured BAL fluid cell counts and differentials in the OVA, OVA-indomethacin, mock, and mock-indomethacin groups of WT, IL-4 KO, IL-4Rα KO, and STAT6 KO mice to determine the effect of COX

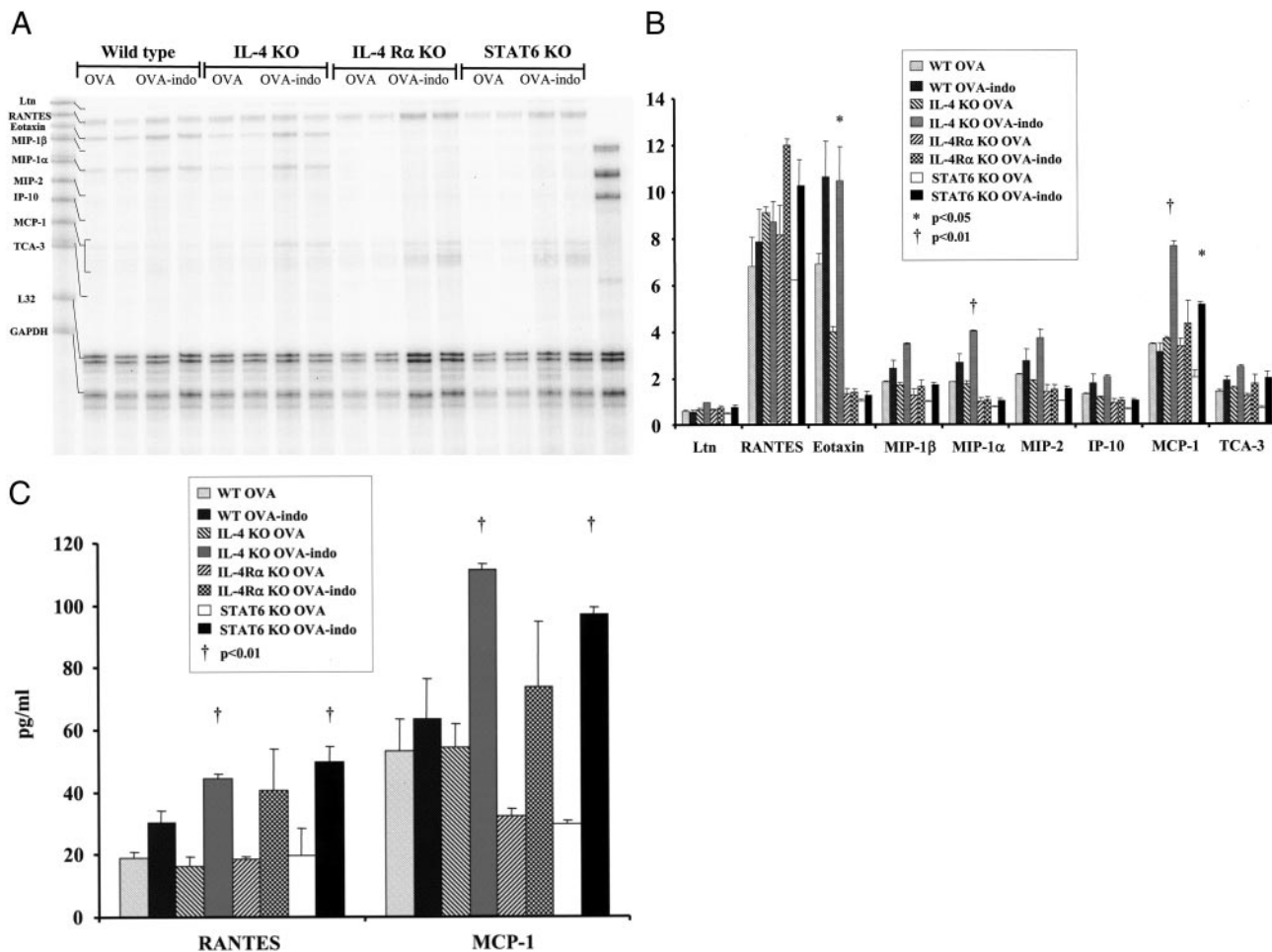


FIGURE 4. A, RPA showing chemokine receptor mRNA present in lungs from WT, IL-4 KO, IL-4R α KO, and STAT6 KO harvested on day 16. B, Data from an RPA showing chemokine mRNA present in lungs harvested on day 16. Data are expressed as mRNA for each cytokine as a percentage of L32. *, $p < 0.05$ compared with the OVA group of either the WT or respective KO mice. C, Concentrations of selected chemokine proteins in ground lung supernatants as measured by protein by laser-based fluorescent analytical testing on day 16 ($n = 4$ –5 for each group). †, $p < 0.01$ compared with the OVA group of the respective KO mice. L32, ribosomal mRNA used as a loading control; indo, indomethacin.

inhibition on cellular recruitment to the airways (Fig. 5). The WT OVA-indomethacin mice had greater total cells (5.7 ± 0.8 vs $2.2 \pm 0.5 \times 10^5$; $p < 0.05$), eosinophils (3.3 ± 0.6 vs $0.6 \pm 0.3 \times 10^5$; $p < 0.05$), and lymphocytes (0.6 ± 0.2 vs $0.1 \pm 0.1 \times 10^5$; $p < 0.05$) than did the WT OVA group or any other group except the IL-4 KO OVA-indomethacin mice. There were more eosinophils in the BAL fluid of the WT and IL-4 KO OVA-indomethacin group than in BAL fluids of any other group ($p < 0.05$), whereas the number of eosinophils in the IL-4R α KO and STAT6 KO mice was greater than all other remaining groups ($p < 0.05$). There were more BAL lymphocytes in the WT, IL-4 KO, IL-4R α KO, and STAT6 KO mice than in any other group ($p < 0.05$). Therefore, the increase in allergic inflammatory cell influx into the airways caused by COX inhibition is independent of STAT6 signaling.

COX inhibition increased mucus production in the OVA-sensitized IL-4 KO mice but not in the IL-4R α KO and STAT6 KO mice

Previously, we reported that abundant mucus is produced in the airway epithelial cells of both OVA-indomethacin and OVA WT mice. We found that in IL-4 KO mice, the OVA-indomethacin group had increased airway epithelial mucus expression mucus compared with OVA mice ($2+ \pm 0$ vs $0.5+ \pm 0.5$; $n = 4$ in each

group). However, there was no airway epithelial mucus present in either the OVA-indomethacin or OVA groups of the IL-4R α KO and STAT6 KO mice (Fig. 6). These results indicate that COX inhibition during the development of allergic lung inflammation can increase mucus independently of IL-4, but mucus production is dependent on signaling through IL-4R α and STAT6. This suggests that IL-13 is critically important in the induction of mucus production.

COX inhibition during allergic sensitization and challenge increases the number of IL-5-producing T lymphocytes in STAT6 KO mice

To define more precisely the source of the type 2 cytokines in the allergically sensitized and challenged WT and STAT6 KO mice, we used intracellular cytokine staining and flow cytometry to analyze the phenotype of the IL-5-producing cells from the lung parenchyma on day 16 (Fig. 7). The number of CD4 $^+$ type 2 cells, those that positively stained for IL-5, was significantly greater in the OVA-indomethacin WT and STAT6 KO groups than in the WT and STAT6 KO OVA, mock-indomethacin, or mock groups. These results reveal that COX inhibition during the development of allergic lung inflammation increases the number of type 2 lymphocytes from STAT6 KO mice and that these lymphocytes are a

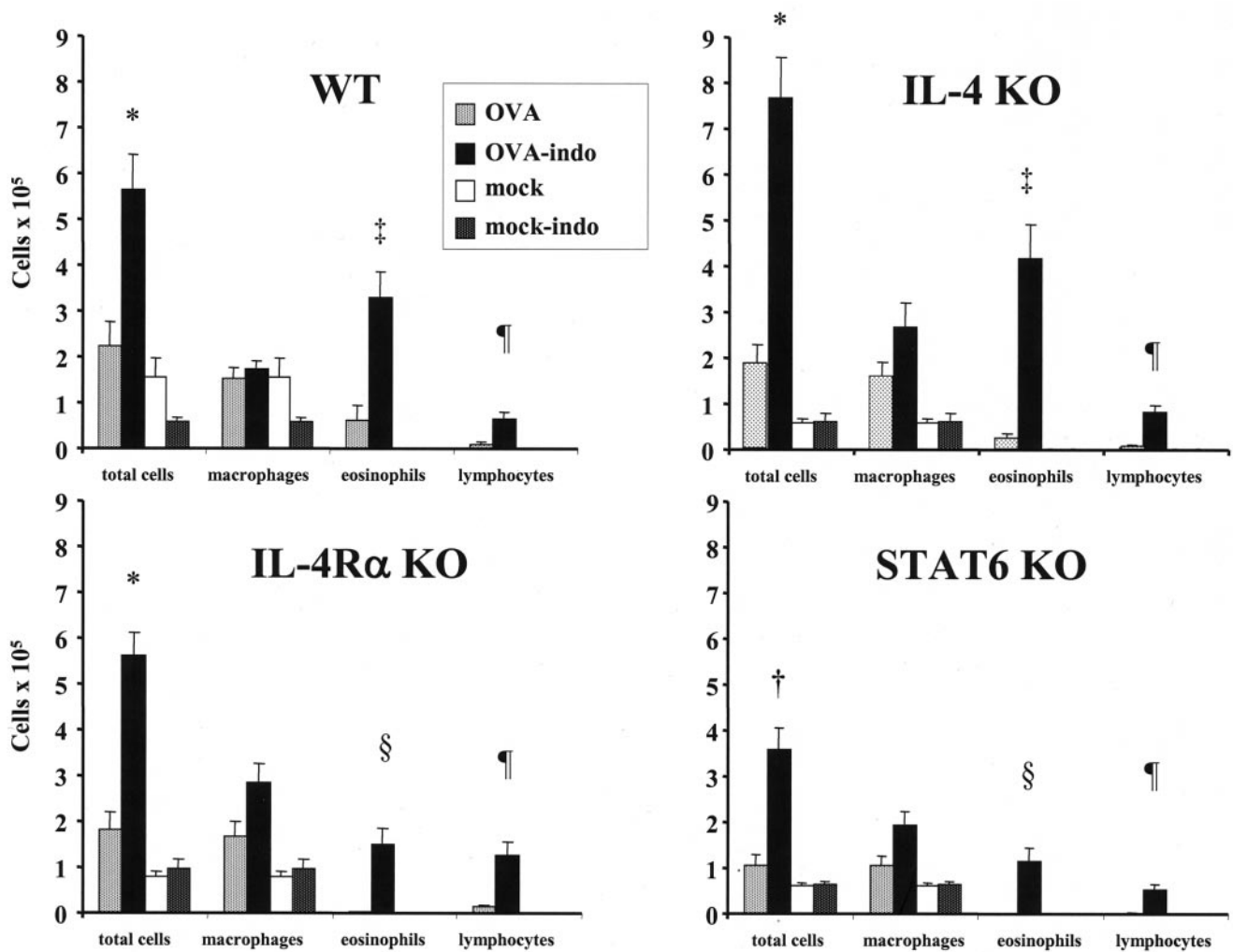


FIGURE 5. Total cells, macrophages, eosinophils, and lymphocytes in the BAL fluid on day 16 from allergically sensitized and nonsensitized WT, IL-4 KO, IL-4R α KO, and STAT6 KO mice treated with either the nonselective COX inhibitor indomethacin (indo) or vehicle in the drinking water. *, $p < 0.05$ compared with the STAT6 KO OVA-indomethacin group and all other OVA, mock, and mock-indomethacin groups. †, $p < 0.05$ compared with all OVA, mock, mock-indomethacin groups except WT OVA. ‡, $p < 0.05$ for all groups except WT or IL-4 KO OVA-indomethacin. §, $p < 0.05$ for all OVA, mock, and mock-indomethacin groups. ¶, $p < 0.05$ for all OVA, mock, and mock-indomethacin groups.

source of the heightened allergic inflammation seen with indomethacin treatment.

COX inhibition increased serum IgE levels in WT mice, but there are undetectable levels in OVA-indomethacin IL-4 KO, IL-4R α KO, or STAT6 KO mice

As COX inhibition increased lung concentrations of IL-13 in the OVA-indomethacin IL-4 KO, IL-4R α KO, and STAT6 KO mice to the levels seen in WT mice, we hypothesized that serum IgE levels would be elevated in these mice as well, as IL-13 has been reported to be a switch factor for IgE synthesis. Although we found that there was a significant increase in serum IgE levels in the WT OVA-indomethacin mice compared with the OVA mice, we did not find that COX inhibition significantly increased IgE levels in the OVA-indomethacin IL-4 KO mice despite the high IL-13 levels in the lungs of these mice (Fig. 8). This suggests that in this model IL-13 alone is not an Ig isotype switch factor for IgE synthesis. Similarly, we found that there were undetectable serum levels of IgE in IL-4R α KO and STAT6 KO mice.

Discussion

Using a murine model of allergic lung disease, we have demonstrated that the increased IL-5 and IL-13 production that occurs with COX inhibition is CD4 cell dependent, but not dependent on signaling through IL-4, IL-4R α , or STAT6. These results indicate that COX inhibition exerts its effect on IL-5 and IL-13 synthesis independently of the signaling pathways that are currently believed to regulate the production of these proteins. COX inhibition also resulted in increased airway eosinophilia and lymphocytosis in allergically sensitized IL-4 KO, IL-4R α KO, and STAT6 KO mice, whereas there was minimal inflammation in these groups of KO mice that had been treated with vehicle. Histopathological analysis revealed that COX inhibition increased epithelial mucus production in IL-4 KO mice, but it did not do so in IL-4R α KO and STAT6 KO mice. COX inhibition also increased serum IgE levels in allergically sensitized WT mice, yet IgE levels were undetectable in allergically sensitized and challenged IL-4 KO, IL-4R α KO, and STAT6 KO mice, which all had high levels of lung IL-13. Our results support that IgE is not necessary for the development of allergic responses in mice (10).

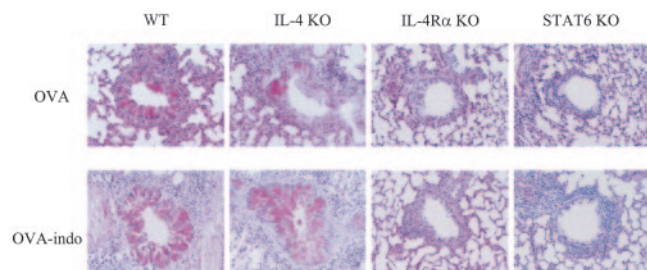


FIGURE 6. PAS staining of lung sections from OVA and OVA-indomethacin (indo) groups for WT, IL-4 KO, IL-4R α KO, and STAT6 KO mice.

Allergic inflammation results from a complex immunological cascade involving the production of the type 2 cytokines IL-4, IL-5, and IL-13 (11). Cells known to produce IL-4, IL-5, and IL-13 include T lymphocytes, mast cells, and basophils (11). To determine whether T lymphocytes were the cellular source of the augmented type 2 cytokines that result from COX inhibition in our murine model, we depleted both CD4 and CD8 cells, because CD8 cells also secrete type 2 cytokines (12). We found that COX inhibition in the setting of T cell depletion did not augment IL-5 and IL-13 production, revealing that the heightened type 2 cytokine production was indeed T cell dependent.

STAT6 is thought to be critical for the induction of IL-4 and IL-13 T lymphocyte responses. STAT6 is activated by the ligation of the IL-4R or IL-13R via phosphorylation by JAK (13). It has previously been reported that T lymphocytes from STAT6 KO mice do not have the capacity to differentiate into type 2 cells in response to IL-4 or IL-13 (14), indicating that the STAT6 pathway has a central role in the allergic response. We confirmed this result by demonstrating that there was no detectable IL-5 or IL-13 by either lung mRNA or cytokine protein in ground lung supernatants in allergically sensitized mice that had received vehicle in their drinking water. However, we found that COX inhibition resulted in WT levels of IL-5 and IL-13 lung mRNA and cytokine protein in STAT6 KO mice, as well as in IL-4 KO and IL-4R α KO mice. These results show that COX is an important regulator of allergic

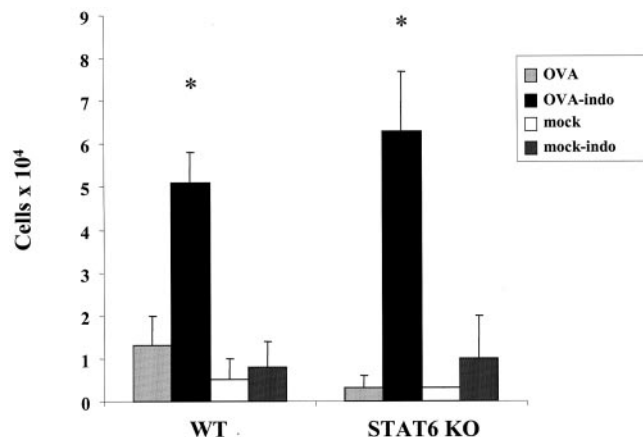


FIGURE 7. Analysis of intracellular cytokine expression in lung lymphocytes on day 16 showing cells stained for CD4 and IL-5 in allergically sensitized and nonsensitized STAT6 KO mice treated with either the non-selective COX inhibitor indomethacin or vehicle in the drinking water ($n = 3$ for the WT and STAT6 KO OVA and OVA-indomethacin groups and $n = 2$ for the WT and STAT6 KO mock and mock-indomethacin (indo) groups). *, $p < 0.05$ compared with both the WT and STAT6 KO OVA, mock, and mock-indomethacin (indo) groups.

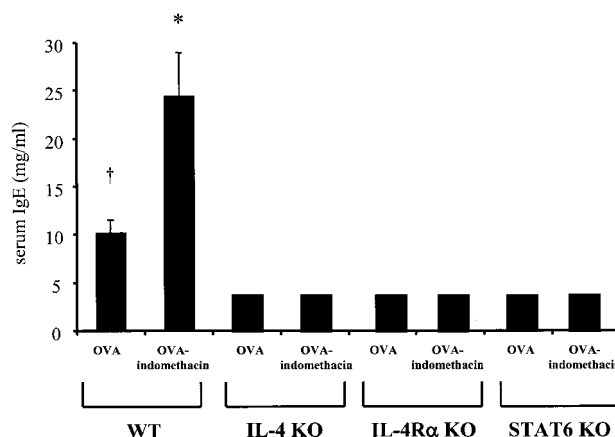


FIGURE 8. Total IgE levels in serum harvested from WT, IL-4 KO, IL-4R α KO, and STAT6 KO at day 16 ($n = 8$). Data are representative of two separate experiments. *, $p < 0.05$ compared with all other groups. †, $p < 0.05$ compared with the OVA and OVA-indomethacin IL-4 KO, IL-4R α KO, and STAT6 KO groups.

inflammation and that it operates, at least partially, through undescribed STAT6-independent pathways.

COX inhibition also altered chemokine expression and augmented inflammatory cell influx into the lung. For instance, COX inhibition during allergic sensitization increased the levels of eotaxin, MIP-1 α , and MCP-1 mRNA in the lungs of IL-4 KO mice. Our results are also consistent with a study showing that IL-13 up-regulates eotaxin expression, independent of IL-4 (15, 16). However, despite the increased IL-13 with COX inhibition in our model, we found that COX inhibition did not up-regulate eotaxin in IL-4R α KO and STAT6 KO mice (17), confirming that eotaxin expression is STAT6 dependent (15, 18).

STAT6 is also an important regulator of CD4 type 2 cell trafficking in allergic pulmonary inflammation. For instance, the phenotype characteristic of allergic asthma is type 2 cell influx into the lung, type 2 cytokine production, pulmonary eosinophilia, and mucus production (18). However, similar allergic inflammation is absent in STAT6 KO mice that are the recipients of polarized type 2 WT cells and are exposed to aerosol Ag challenge, results suggesting that STAT6 is essential for type 2 trafficking and effector function (18). Our results using vehicle-treated drinking water confirm that STAT6 is essential in the development of allergic lung inflammation; however, COX inhibition allowed for STAT6-independent development of eosinophil and lymphocyte cellular influx into the airway. In addition, the number of CD4 IL-5-producing cells in the lung was significantly increased in the allergically sensitized STAT6 KO mice treated with the COX inhibitor, whereas those allergically sensitized STAT6 KO mice treated with vehicle had the same number of lung type 2 cells as nonsensitized mice. These findings, along with our CD4 and CD8 Ab depletion studies, confirm the critical influence of T lymphocytes in the augmented allergic inflammation that occurs with COX inhibition.

Exogenous administration of IL-13 in WT mice results in increased serum IgE levels, and IL-13 is reportedly an isotype switch factor for the production of IgE (19). We found that serum IgE levels were significantly increased in COX inhibitor-treated allergically sensitized WT mice that were and that IL-13 levels in the lung were augmented. However, COX inhibition increased IL-13 in the lungs of allergically sensitized IL-4 KO to WT levels, whereas there was no detectable serum IgE in these mice. This suggests that in our model, IL-13 alone is not an isotype switch

factor for IgE production and that IL-4 is necessary for IgE production. Exogenous administration of IL-13 may increase IgE levels in the presence of existing IL-4 or may increase IL-4 levels which in turn increase IgE levels. However, others have reported IgE induction in IL-4 KO mice. For instance, transgenic mice expressing IL-13, yet that are IL-4 deficient, produce IgE (20). In addition, IgE synthesis has been reported to occur in both IL-4 KO and IL-4R α KO exposed to 6 wk, but not 3 wk, of allergen (21).

The COX products that are responsible for the suppression of allergic inflammation in STAT6 KO mice are currently unknown. Although in vitro studies suggest that PGE₂ may enhance allergic inflammation (22–25), an in vivo study in which PGE₂ was administered acutely before allergen challenge suppressed the migration of IL-4 and IL-5 mRNA-expressing cells in BAL fluid (26). In addition, mice that lack the PGI₂ receptor, i.p., have increased allergic inflammation compared with WT mice (27). These results suggest that PGE₂ and PGI₂ may restrain allergic responses in the lung. Recently, Zimmerman et al. (28) profiled expression of genes that were altered in lung tissue in a murine allergen challenge model similar to ours. By microarray data analysis, gene transcript levels were determined in WT mice that were either saline or allergen challenged. These investigators found that expression of the PGE₂ receptor 4 and i.p. were both increased with allergic inflammation (28). We are investigating the possibility that PGE₂ and/or PGI₂ restrain allergic inflammation in STAT6 KO mice.

Overall, our findings establish that COX is a master regulator of allergic inflammation. COX controls type 2 cytokine generation through its activity on T lymphocytes and also controls eosinophil and lymphocyte trafficking. In addition, we found that IL-4 is the necessary IgE isotype switch factor and that it is also under the control of COX regulation.

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