

Allergen-Induced Airway Hyperresponsiveness Mediated by Cyclooxygenase Inhibition Is Not Dependent on 5-Lipoxygenase or IL-5, but Is IL-13 Dependent¹

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Cyclooxygenase (COX) inhibition during allergic sensitization and allergen airway challenge results in augmented allergic inflammation. We hypothesized that this increase in allergic inflammation was dependent on increased generation of leukotrienes that results from COX inhibition, as leukotrienes are important proinflammatory mediators of allergic disease. To test this hypothesis, we allergically sensitized and challenged mice deficient in 5-lipoxygenase (5-LO). We found that 5-LO knockout mice that were treated with a COX inhibitor during allergic sensitization and challenge had significantly increased airway hyperresponsiveness (AHR) ($p < 0.01$) and airway eosinophilia ($p < 0.01$) compared with 5-LO knockout mice that were treated with vehicle. The proinflammatory cytokines have also been hypothesized to be critical regulators of airway inflammation and AHR. We found that the increase in airway eosinophilia seen with COX inhibition is dependent on IL-5, whereas the increase in AHR is not dependent on this cytokine. In contrast, the COX inhibition-mediated increase in AHR is dependent on IL-13, but airway eosinophilia is not. These results elucidate the pathways by which COX inhibition exerts a critical effect of the pulmonary allergen-induced inflammatory response and confirm that COX products are important regulators of allergic inflammation. *The Journal of Immunology*, 2005, 175: 8253–8259.

During allergic sensitization and allergen airway challenge, cyclooxygenase (COX)⁴ inhibition results in augmented allergic inflammation (1–4). This increase in the allergic phenotype occurs when mice are treated with a nonselective COX inhibitor or with pharmacologic agents that specifically inhibit COX-1 or COX-2 (1–4). This up-regulation of the allergen-induced pulmonary inflammatory response by COX inhibition is independent of signaling through the IL-4R or the STAT6 transcription factor (1). Additionally, COX inhibition results in augmented allergen-induced airway hyperresponsiveness (AHR) (2–4).

Currently, it is not known what factors regulate the heightened allergic phenotype that is driven by COX inhibition. Arachidonic acid can be metabolized not only through the COX enzymes, but also through the 5-lipoxygenase (5-LO) pathway, an important source of proinflammatory mediators (5). Leukotriene (LT)_{A₄} can be metabolized by either LTA₄ hydrolase into LTB₄, which is a

potent neutrophil chemoattractant and a weaker eosinophil chemotactic agent, or by LTC₄ synthase into the cysteinyl LT (5). The cysteinyl LT have eosinophil chemotactic properties, are potent mucus secretagogues, and induce AHR (6). We have previously shown that COX inhibition in the murine model of allergic pulmonary inflammation results in increased cysteinyl LT levels (3). Recent studies suggest that signaling through the LTB₄ receptor 1 is important in allergen-induced AHR (7, 8). Therefore, we hypothesized that the increase in LT resulting from COX inhibition mediates the augmented allergic phenotype in our model.

Cytokines are also important regulators of allergic inflammation and AHR. IL-5 is a critical factor in eosinophil development, differentiation, mobilization, activation, and survival (9–13). Animal models of allergic sensitization in which IL-5 has either been neutralized by Ab, or in which the gene producing IL-5 has been deleted, suggest that IL-5 is critical to the control of airway eosinophilia (14–16). The role of IL-5 in mediating AHR is less clear, as some investigators have found that IL-5 is necessary for AHR (16–19), whereas others have found that AHR can occur independently of IL-5 (20–22). Mouse studies examining the role of IL-13 suggest that it is necessary and sufficient to induce the allergic phenotype, including AHR (23–25). Experimental work in the murine model reveals that IL-13 acts on the resident cells in the airway to induce mucus secretion and AHR, rather than through recruitment of eosinophils to the lung or through type I-mediated hypersensitivity (25). Therefore, we also sought to determine whether the allergen-induced airway inflammation and AHR that occurs with COX inhibition was dependent on either IL-5 or IL-13.

Materials and Methods

Mice

Pathogen-free 8- to 10-wk-old female mice were used in all experiments. The 5-LO knockout (KO) mice and their wild-type (WT) control mice were on a 129 genetic background and were purchased from The Jackson

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Received for publication June 23, 2005. Accepted for publication September 29, 2005.

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¹ This work was supported by the American Academy of Allergy, Asthma and Immunology Education and Research Trust Grants and Awards HL-069949, AI 054660, GM 15431, DK48831, and CA77839.

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⁴ Abbreviations used in this paper: COX, cyclooxygenase; 5-LO, 5-lipoxygenase; AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; indo, indomethacin; KO, knockout; LT, leukotriene; WT, wild type; PC, provocative concentration.

Laboratory. The IL-5 KO mice and their WT control mice were on a C57BL/6 genetic background and were purchased from The Jackson Laboratory. IL-13 KO mice were generated as previously described and had been bred back to a C57BL/6 genetic background (26). In caring for animals the investigators adhered to the revised 1996 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.

Allergen sensitization and challenge protocol

Mice were allergically sensitized by an i.p. injection of 0.1 ml (10 μ g) of OVA (chicken OVA, grade V; Sigma-Aldrich) complexed with 20 mg of $\text{Al}(\text{OH})_3$ diluted in a volume of 100 μ l of PBS on day 0. On days 14 through 17, the mice were challenged by placing them in an acrylic box and exposing them to aerosols of 1% OVA diluted in sterile PBS using an ultrasonic nebulizer (Ultraneb 99; DeVilbiss) for 40 min each day. Non-sensitized mice were injected i.p. with 20 mg of $\text{Al}(\text{OH})_3$ diluted in a volume of 100 μ l of PBS on day 0.

COX inhibitor administration

Indomethacin (30 μ g/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 was added to 200 ml of water in the animal water bottles. The water of the vehicle-treated mice was also changed three times per week and 2 ml of ethanol was added to 200 ml of water in the water bottles of those mice. Mice that were allergically sensitized and challenged and treated with indomethacin are designated OVA-*indo*. Mice that were allergically sensitized and challenged and treated with vehicle are designated OVA. Mice that were not sensitized and treated with indomethacin are designated mock-*indo*. Mice that were not sensitized and treated with vehicle are designated mock. We have shown that this method of indomethacin administration significantly reduces PGE_2 levels in bronchoalveolar lavage (BAL) fluid in the OVA sensitization and challenge model (4).

Methacholine challenge

Mice were anesthetized with i.p. injections of pentobarbital sodium (85 mg/kg) and a tracheostomy tube was placed. The internal jugular vein was cannulated and a microsyringe was attached to i.v. tubing for methacholine administration. The mice were then placed in a whole body plethysmography chamber and mechanically ventilated. Lung resistance was measured following administration of i.v. acetyl- β -methacholine (Sigma-Aldrich) as previously described (27). Methacholine dose-response curves were obtained by calculating the mean $\pm$ SE for individual animals at each methacholine dose. The provocative concentration dose of methacholine that led to a 200% increase in baseline lung resistance (PC_{200}) is expressed as mean $\pm$ SE for individual animals.

BAL analysis

Following lung resistance measurements, the animals were given a lethal injection of pentobarbital. BAL was then performed by instilling 600 μ l of 5% BSA diluted in normal saline through the tracheostomy tube and then withdrawing the fluid with gentle suction via the syringe. The typical BAL fluid return was 300–400 μ l. White blood cells were counted on a hemocytometer. Cytologic examination was performed on cytospin preparations (Thermo Shandon). Cytospin slides were fixed and stained using DiffQuik (American Scientific Products). Differential counts were based on counts of 100 cells using standard morphologic criteria to classify the cells as eosinophils, lymphocytes, or other mononuclear leukocytes (alveolar macrophages and monocytes).

Measurement of cytokine proteins

Lung tissues were ground as previously described (4), and the levels of IL-5 and IL-13 in the lung tissue homogenates were measured in the 129 WT and 5-LO KO mice with a commercially available LINCplex mouse cytokine/chemokine kit (Linco Research) using fluorescent-labeled microsphere beads and a Luminex reader. Cytokine levels were measured in the C57BL/6 WT and IL-5 KO mice using ELISA kits following the manufacturer's instructions (R&D Systems). The experiments using 5-LO KO and 129 mice were performed more recently and the newer fluorescent-labeled microsphere bead technology was used in these experiments.

IgE concentrations

Before sacrifice on day 18, sera were collected from sensitized mice, then analyzed by ELISA to determine levels of total IgE as previously described (1). OVA-specific IgE levels were also measured by ELISA. In this protocol, 96-well Immunolon II plates (Nunc) were coated with 100 μ l of OVA dissolved in sterile PBS at a concentration of 20 μ g/ml. 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 μ l of serum diluted 1/200 in PBS/0.1% BSA/0.05% Tween 20 in duplicate. Plates were incubated overnight and washed, and 100 μ l of rat anti-mouse IgE clone LO-ME-2 (Serotec) diluted 1/2000 was added to each well. After 1 h 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 measured at 450 nm (OD_{450}). The value reported for the OVA-specific IgE concentration for each mouse is the average of duplicate OD_{450} values.

Statistical analysis

Results are expressed as mean $\pm$ SEM. Measurements of cytokines by ELISA, cell counts and differentials, PC_{200} , and IgE by ELISA were analyzed by ANOVA with Fisher's least significant difference performed as a post hoc analysis. Dose-response curves to methacholine were compared by repeated measures by ANOVA with Fisher's least significant difference performed as a post hoc analysis. Differences were considered to be significant if values for $p < 0.05$.

Results

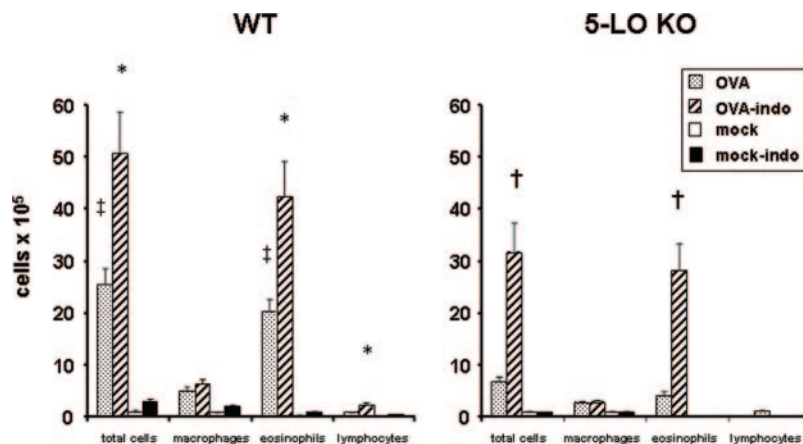
COX inhibition increased allergen-induced cellularity of BAL in 5-LO KO mice

Because cysteinyl LT are important mediators in allergic inflammation, we measured BAL fluid cell counts and differentials in the OVA and OVA-*indo* groups of WT and 5-LO KO mice to determine whether the increase in LT was responsible for the increased allergic inflammation in indomethacin-treated mice. The WT OVA-*indo* mice had greater total cells (50.6 ± 7.9 vs $25.3 \pm 3.1 \times 10^5$; $p < 0.01$), eosinophils (42.3 ± 6.9 vs $20.2 \pm 2.3 \times 10^5$; $p < 0.01$), and lymphocytes (2.0 ± 0.6 vs $0.6 \pm 0.1 \times 10^5$; $p < 0.01$) in the BAL fluid than did the WT OVA group or any other group (Fig. 1). There were more total cells and eosinophils in the BAL fluid of the 5-LO KO OVA-*indo* groups and WT OVA group than in BAL fluids of the remaining groups, including the 5-LO KO OVA group ($p < 0.01$). Therefore, the increase in allergic inflammatory cell influx into the airways caused by COX inhibition is not fully dependent on the expression of 5-LO.

COX inhibition increased AHR in allergen-sensitized and -challenged 5-LO KO mice

Methacholine challenge was performed on day 18, one day after the last OVA aerosol treatment in both WT and 5-LO KO mice. The AHR was significantly greater in the 5-LO KO OVA-*indo* mice compared with any other 5-LO KO group ($p < 0.05$), whereas the AHR of the 5-LO KO OVA mice was no different from the 5-LO KO mock and mock-*indo* groups (Fig. 2). The AHR was significantly greater in the WT OVA-*indo* group compared with any other group of WT or 5-LO KO mice ($p < 0.05$), whereas the AHR of the WT OVA mice was greater than the AHR of the WT mock and mock-*indo* groups ($p < 0.05$), and no different from the 5-LO KO OVA-*indo* group. The PC_{200} values, reported as micrograms of methacholine per gram of mouse, were 552 ± 184 for WT mock, 1039 ± 650 for WT mock-*indo*, 170 ± 21 for WT OVA, and 162 ± 3 for WT OVA-*indo*, and there were no differences between the groups. The PC_{200} values were 2792 ± 461 for 5-LO KO mock, 1460 ± 534 for 5-LO KO mock-*indo*, 1801 ± 413 for 5-LO KO OVA, and 678 ± 281 for 5-LO KO OVA-*indo*, with values in the 5-LO KO OVA-*indo* group being significantly

FIGURE 1. Total cells, macrophages, eosinophils, and lymphocytes in the BAL fluid on day 18 from allergically sensitized and challenged or nonsensitized WT and 5-LO KO mice treated with either the nonselective COX inhibitor indomethacin (indo) or vehicle in the drinking water ($n = 9$ –10 for all OVA and OVA-indo groups and $n = 6$ –8 for all mock and mock-indo groups). Statistical significance is indicated by *, $p < 0.01$ compared with all other groups; †, $p < 0.01$ compared with all other groups except WT OVA-indo and WT OVA; and ‡, $p < 0.01$ compared with all other groups except WT OVA-indo and 5-LO KO OVA-indo. The results are representative of three separate experiments.



lower than those in 5-LO KO mock ($p < 0.01$) and 5-LO KO OVA ($p < 0.05$) groups.

The augmented IL-5 and IL-13 production resulting from COX inhibition is dependent on 5-LO

Previously, we found that the levels of IL-5 in ground lung supernatants peaked on day 16 of our protocol; therefore, we measured cytokines on this day. We found that IL-5 (767 ± 218 vs 200 ± 126 pg/ml; $p < 0.01$) and IL-13 (449 ± 105 vs 167 ± 61 pg/ml; $p < 0.01$) concentrations were greater in the lung supernatants of the WT OVA-indo group compared with the WT OVA group (Fig. 3). However, there was only a trend for an increase in IL-5 (276 ± 123 vs 63 ± 8 pg/ml; $p = 0.06$) and IL-13 (225 ± 67 vs 100 ± 5 pg/ml; $p = 0.1$) in the 5-LO KO OVA-indo group compared with the 5-LO KO OVA group that was not statistically significant. This suggests that the increase in Th2 cytokines that occurs with COX inhibition during the development of allergic inflammation is dependent on 5-LO.

Elevated IgE levels resulting from COX inhibition is dependent on 5-LO

Because the WT OVA-indo and 5-LO KO OVA-indo mice had greater numbers of inflammatory cells in the BAL fluid compared with the rest of the mice in their respective groups, we sought to

determine whether this might be a result of an effect of COX inhibition on serum IgE levels (Fig. 4). We found that the WT OVA-indo mice had greater serum concentrations of IgE compared with WT OVA mice (30.3 ± 2.7 vs 13.7 ± 1.9 μ g/ml, respectively; $p < 0.01$). However, there was no difference in the serum IgE levels between the 5-LO KO OVA-indo and 5-LO KO OVA mice as there were undetectable serum IgE levels in all the 5-LO KO OVA mice and undetectable levels in four of the eight 5-LO KO OVA-indo mice. We also measured OVA-specific IgE levels in the serum of the mice and the results are reported as the OD₄₅₀ as stated in *Materials and Methods*. The WT OVA-indo mice had significantly greater serum concentrations of OVA-specific IgE compared with WT OVA mice (304 ± 26 vs 91 ± 15 OD₄₅₀; $n = 6$ in each group; $p < 0.0001$). OVA-specific IgE levels were undetectable in the 5-LO OVA-indo and 5-LO OVA mice. Therefore, this suggests that the increase in serum IgE levels seen with COX inhibition is dependent on the presence of 5-LO.

COX inhibition mediated increase in BAL eosinophils is dependent on IL-5, but not IL-13

IL-5 is a critical cytokine in eosinophil growth, differentiation, and survival; however, IL-13 has also been reported to be a factor in eosinophil recruitment to the airways (23). We therefore wanted to determine whether the increased airway eosinophilia seen with COX inhibition during the development of allergic pulmonary inflammation was mediated by IL-5 or IL-13. In these experiments, we used WT, IL-5 KO, and IL-13 KO mice, all on a C57BL/6 background. We found that WT OVA-indo mice had increased

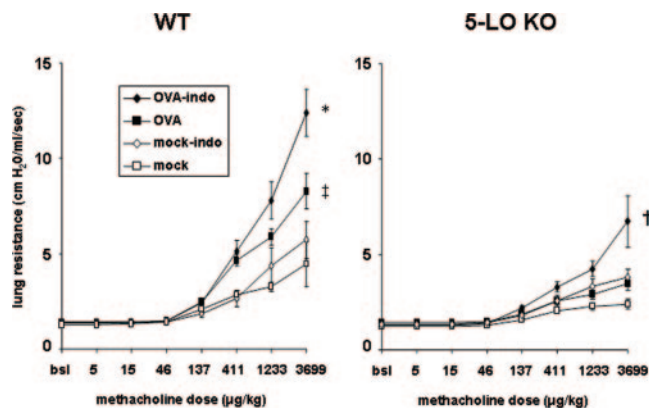


FIGURE 2. Results from methacholine challenge performed on day 18. The data shown are a combination of two experiments ($n = 14$ –18 for OVA-indo and OVA; $n = 5$ –8 for mock-indo and mock). Data points represent the mean $\pm$ SEM measuring lung resistance. Statistical significance is indicated by *, $p < 0.05$ compared with all other groups; †, $p < 0.01$ compared with all other groups except WT OVA-indo and WT OVA; and ‡, $p < 0.01$ compared with all other groups except WT OVA-indo and 5-LO KO OVA-indo.

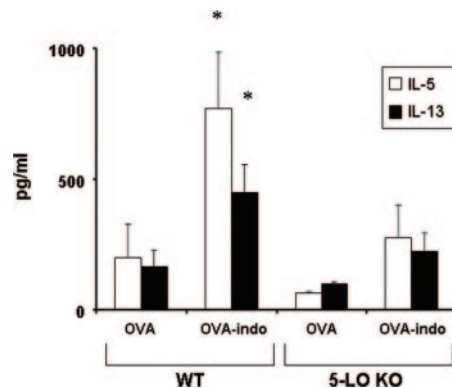


FIGURE 3. Concentrations of IL-5 and IL-13 in the lung supernatants from WT and 5-LO KO mice on day 16 ($n = 4$ –5 for each group). Data points represent the mean $\pm$ SEM and are representative of three separate experiments. *, $p < 0.01$ compared with all other groups.

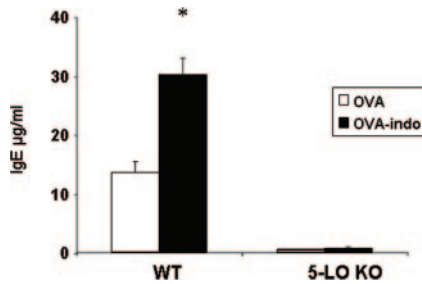


FIGURE 4. Total IgE levels in serum harvested from WT and 5-LO KO mice at day 18 ($n = 8$ for each group). Data are representative of three separate experiments. *, $p < 0.01$ compared with all other groups.

total cells (20.4 ± 4.5 vs $8.8 \pm 2.5 \times 10^5$; $p < 0.05$) and eosinophils (15.4 ± 3.4 vs $5.6 \pm 2.0 \times 10^5$; $p < 0.05$) in BAL fluid compared with WT OVA mice (Fig. 5). In addition, we also found that a similar number of total cells and eosinophils in the BAL fluid of IL-13 KO OVA-Indo mice compared with WT OVA-Indo mice, but the IL-13 KO OVA-Indo mice had significantly greater total cells (25.0 ± 5.8 vs $3.9 \pm 0.6 \times 10^5$; $p < 0.05$) and eosinophils (16.8 ± 4.7 vs $1.5 \pm 0.5 \times 10^5$; $p < 0.05$) in BAL fluid compared with the IL-13 KO OVA group. There was no difference in the total cells (3.4 ± 1.1 vs $1.7 \pm 0.4 \times 10^5$) or eosinophils (0.4 ± 0.1 vs $0.4 \pm 0.3 \times 10^5$) in the IL-5 KO OVA-Indo mice compared with the IL-5 KO OVA mice, and both of these groups had significantly fewer total cells and eosinophils compared with either the WT OVA-Indo or IL-13 KO OVA-Indo mice. Thus the increase in BAL eosinophils seen with COX inhibition during the development of allergic inflammation is mediated by IL-5, and not IL-13.

The increased AHR seen with COX inhibition is dependent on IL-13, but not IL-5

To determine whether the mechanism that led to an increase in AHR seen with COX inhibition was driven by a cytokine that has been reported to be important in AHR, we again used WT, IL-5 KO, and IL-13 KO mice, all on a C57BL/6 background. We found that there was no difference in AHR between WT OVA, WT mock-Indo, and WT mock mice; however, the WT OVA-Indo group had significantly greater AHR ($p < 0.05$) compared with the other three groups (Fig. 6a). We also found similar results in the IL-5 KO mice. There was no difference in AHR between the IL-5 KO OVA, mock-Indo IL-5 KO OVA, and mock IL-5 KO OVA mice; however, the IL-5 KO OVA-Indo group had significantly greater AHR ($p < 0.05$) compared with the other three groups (Fig. 6b). In contrast, we found there were no differences in AHR among the IL-13 KO OVA-Indo mice, IL-13 KO OVA mice, IL-13 KO mock-Indo mice, and IL-13 KO mock mice (Fig. 6c).

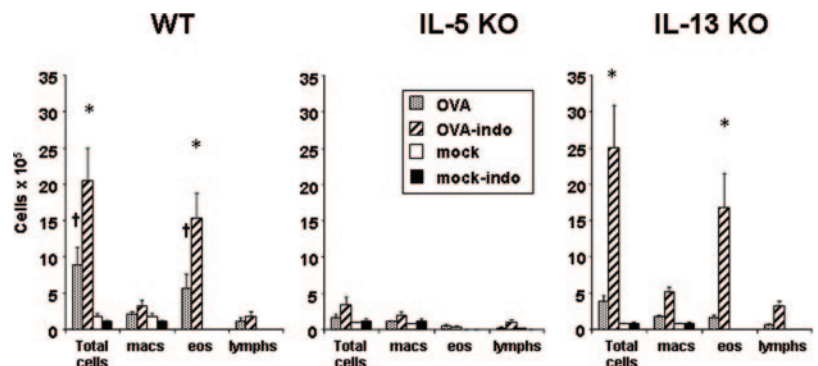
These results show, that despite an increase in BAL cell influx in the IL-13 KO OVA-Indo mice, the allergen-induced increase in AHR with COX inhibition was IL-13 dependent.

We also calculated the PC_{200} of methacholine for all the WT, IL-5 KO, and IL-13 KO groups. For the C57BL/6 WT mice, the PC_{200} values, reported as micrograms of methacholine per gram of mouse, were 405 ± 6 for WT mock, 347 ± 28 for WT mock-Indo, 244 ± 39 for WT OVA, and 179 ± 51 for WT OVA-Indo. The PC_{200} values for the WT OVA-Indo and WT OVA groups were each lower than either the WT mock or WT mock-Indo ($p < 0.05$), but were not different from each other. For the IL-5 KO mice, the PC_{200} values were 387 ± 24 for the IL-5 KO-mock, 287 ± 58 for the IL-5 KO-Indo, 281 ± 44 for IL-5 KO OVA, and 160 ± 37 for IL-5 KO OVA-Indo. The IL-5 KO OVA-Indo group had a significantly lower PC_{200} compared with both the IL-5 KO OVA and IL-5 KO mock groups ($p < 0.05$), but there were no differences between the other groups. For the IL-13 KO groups, the PC_{200} value was greater than $411 \mu\text{g/kg}$ for all mice, except for two of eight in the IL-13 KO OVA-Indo group. There was no statistical difference in PC_{200} between the IL-13 KO groups.

COX inhibition increases IL-13 in WT and IL-5 KO mice, and IL-5 in IL-13 KO mice, all on a C57BL/6 background

As AHR was increased in the WT OVA-Indo and IL-5 KO OVA-Indo mice compared with these strains' respective OVA groups, yet AHR was not increased in the IL-13 KO OVA-Indo group, we sought to determine whether IL-13 levels in the lung were increased in the WT OVA-Indo and IL-5 KO OVA-Indo mice. Cytokine proteins were measured in the ground lung supernatants on day 16 (Fig. 7). IL-5 was not detectable in the lung supernatants of IL-5 KO mice, nor was IL-13 detectable in the lung supernatants of the IL-13 KO mice. However, in WT mice, there was a trend ($p = 0.1$) for an increase in IL-5 in the OVA-Indo mice (36.4 ± 15 pg/ml) compared with the OVA mice (<15.6 pg/ml, limit of detection). IL-13 levels in the lung supernatants of the IL-5 KO OVA-Indo group were 174 ± 46 pg/ml compared with undetectable (<31.2 pg/ml) in the IL-5 KO OVA group ($p < 0.05$). In WT mice, the OVA-Indo group had significantly greater levels of IL-13 in the lung supernatants compared with the WT OVA group (110 ± 17 vs 28.4 pg/ml; $p < 0.05$). Levels of IL-13 were not detected in the lung supernatants of either nonsensitized IL-5 KO mice or nonsensitized WT mice, whether treated with indomethacin or not. There was significantly greater IL-5 levels in the lungs of the IL-13 KO OVA-Indo group compared with the IL-13 KO OVA mice (171 ± 28 vs <15.6 pg/ml, limit of detection; $p = 0.001$).

FIGURE 5. Total cells, macrophages (macs), eosinophils (eos), and lymphocytes (lymphs) in the BAL fluid on day 18 from allergically sensitized and nonsensitized WT, IL-5 KO, and IL-13 KO mice either treated with the nonselective COX inhibitor indomethacin (Indo) or vehicle in the drinking water. Statistical significance is indicated by *, $p < 0.05$ compared with the respective OVA, mock, and mock-Indo groups for that particular strain and †, $p < 0.05$ compared with the respective mock and mock-Indo groups for that particular strain.



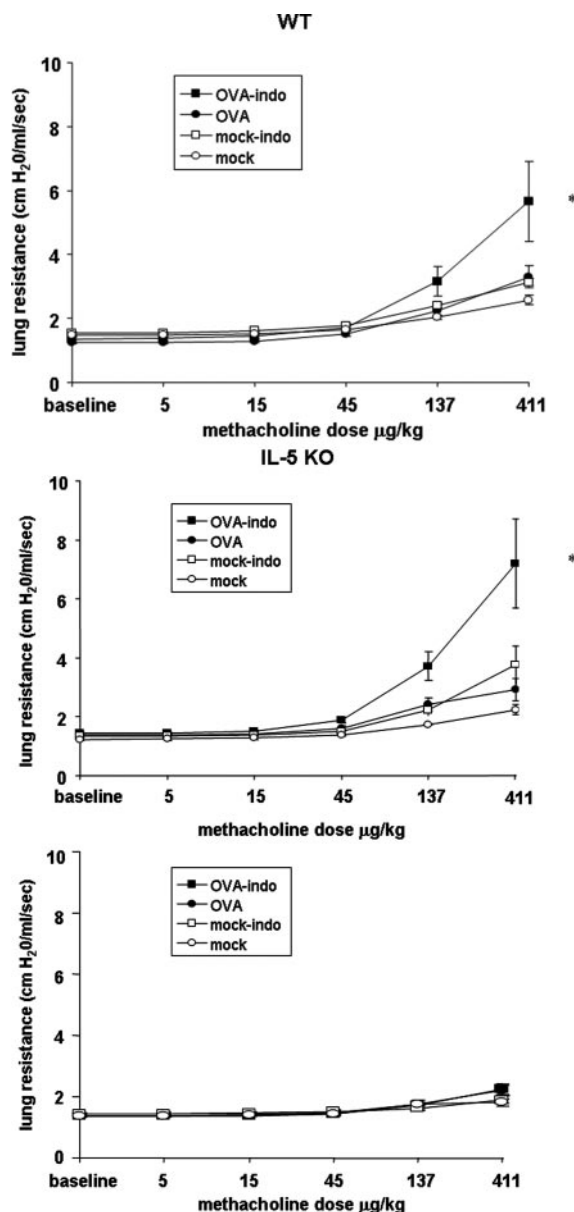


FIGURE 6. Results from methacholine challenge performed on day 18. *a*, In the WT mice ($n = 6$ –9 mice per group) data are representative of two separate experiments. *b*, Data shown for IL-5 KO are combined data from two experiments ($n = 11$ for OVA, $n = 12$ for OVA-indo, $n = 5$ from mock, $n = 6$ for mock-indo). *c*, In IL-13 KO mice ($n = 7$ –9 for the OVA and OVA-indo groups and 4–6 for the mock and mock-indo groups), data are representative of two separate experiments. Data points represent the mean $\pm$ SEM measuring lung resistance. *, $p < 0.05$ compared with the OVA, mock-indo, and mock groups.

Discussion

In our previous studies, we found that COX inhibition up-regulated the allergic phenotype in a mouse model of allergen-induced inflammation (1, 3, 4). Others have found that deficiency of COX-1, but not COX-2 deficiency, results in increased Th2 cytokine production, AHR, and LT generation (28, 29). COX inhibition enhances the production of LT, presumably by decreasing PGE₂, a mediator that negatively regulates LT synthesis (30, 31). An alternative theory is that COX inhibition prevents prostanoid formation and that the increased arachidonic acid substrate is therefore “shunted” toward LT synthesis. However, there is experimental evidence suggesting that shunting does not occur (32). Murine

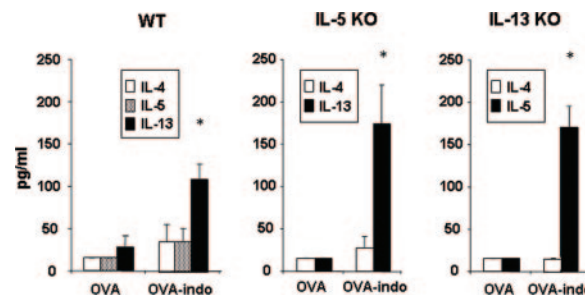


FIGURE 7. Concentrations of IL-5 and IL-13 in the lung supernatants from WT and IL-5 KO mice on day 16 ($n = 4$ –5 for each group). Data points represent the mean $\pm$ SEM and are representative of three separate experiments. *, $p < 0.01$ compared with all other groups.

models reveal that LT are important regulators of pulmonary allergic inflammation. Mice lacking a functioning 5-LO enzyme had significantly decreased airway eosinophilia, serum IgE levels, and AHR compared with WT mice (33). Specific inhibitors of 5-LO and 5-LO-activating protein decreased airway and lung eosinophilia and airway mucus expression (34). Therefore, an important question to be answered was whether the increase in allergic inflammation that occurs with COX inhibition seen in our model is a result of an increase in LT generation. We found that the COX inhibition-induced increase in allergic inflammation and AHR was not solely dependent upon LT expression, as the 5-LO KO OVA-indo mice had significantly increased airway inflammation and AHR compared with the increase for the 5-LO KO OVA group. We reported AHR by two methods: 1) dose-response curves of lung resistance to increasing doses of methacholine and 2) the PC₂₀₀. The changes in AHR as measured by the methacholine dose-response curve and the PC₂₀₀ were not entirely consistent. This is a reflection of the fact that the differences between groups observed in the methacholine dose-response curves occurred at higher doses of methacholine. Both the methacholine dose-response curves and PC₂₀₀ values are measures of AHR and one is not necessarily more valid than the other. In this report, we use the differences in the methacholine dose-response curves to define AHR.

The increases in airway eosinophilia and AHR in the 5-LO KO OVA-indo mice were not as great as was seen in the WT OVA-indo mice, thus not ruling out the possibility that LT may be playing some role in the COX inhibition-mediated enhancement of allergic inflammation. Lung IL-5 and IL-13 expression was significantly increased in the WT OVA-indo mice compared with the WT OVA group, and although lung IL-5 and IL-13 expression was 3- and 2-fold greater in the 5-LO KO OVA-indo mice compared with the 5-LO KO OVA group, these differences were not statistically significant, suggesting that regulation of these cytokines may be partially dependent on 5-LO expression. There was no difference in total serum IgE levels in the 5-LO KO OVA-indo and 5-LO KO OVA mice, whereas there was a significant increase in the WT OVA-indo group compared with the WT OVA mice, suggesting that the COX inhibition-mediated increase in serum IgE is dependent on the expression of 5-LO.

Because the augmentation of allergic inflammation by COX inhibition is not solely dependent on increased LT synthesis, this suggests that a COX product restrains allergic inflammation independently of an effect on LT expression. Murine models suggest that several prostanoids may regulate allergic inflammation. Mice lacking the PGE₂ receptor EP3 had increased allergic inflammation compared with either WT mice or mice lacking each of the other three EP receptors (35), suggesting that signaling through

EP3 down-regulates allergic inflammation. Mice that are deficient in the PGI₂ receptor IP had enhanced allergic inflammation, both in acute and chronic models of allergen-induced pulmonary disease (36, 37), suggesting that PGI₂ also suppresses the allergen-induced inflammatory response. In contrast, in *in vivo* models, there is the suggestion that PGD₂ up-regulates the allergen-induced inflammatory response. Mice lacking the PGD₂ receptor DP had diminished pulmonary allergic inflammation (38), whereas mice that overexpress PGD₂ synthase had augmented an augmented allergic phenotype (39). Studies using either a thromboxane synthase inhibitor or a thromboxane receptor antagonist decreased allergic inflammation (40), suggesting that this mediator also enhances allergic inflammation in the lung. Given that the overall effect of inhibiting COX increases allergic inflammation, it is likely that the prostanoids that restrain allergic inflammation have greater activity than those that increase the allergic phenotype.

Cytokines are also very important mediators of allergic inflammation. We previously found that the increase in the pulmonary airway eosinophilia modulated by COX inhibition was independent of signaling through either the IL-4R α or STAT6 (1). However, the pathway by which COX inhibition increases airway eosinophilia is unknown. IL-5 is a critical factor in eosinophil development, differentiation, mobilization, activation, and survival (9–13). *In vitro*, IL-5 primes eosinophils to respond to eotaxin and the ability of eotaxin to mediate eosinophil chemotaxis is augmented by increased levels of IL-5 (41). We found that the increase in airway eosinophilia was dependent on IL-5 expression. In WT OVA-*indo* mice on a C57BL/6 background, there was a significant increase in airway inflammation and eosinophilia compared with WT OVA mice, which was absent in IL-5 KO OVA-*indo* mice. We found that the COX inhibition driven increase in airway eosinophils was not dependent on IL-13, as IL-13 KO OVA-*indo* mice had significantly greater airway inflammation compared with IL-13 KO OVA mice and was comparable to the inflammation seen in WT OVA-*indo* mice. This finding would be consistent with the report that IL-13 induced eosinophil recruitment into the lung by an IL-5 and eotaxin-dependent mechanism (42).

Although we found that the increase in airway eosinophilia resulting from COX inhibition was dependent on IL-5, we found that the increase in allergen-induced AHR mediated by COX inhibition was not dependent on this cytokine, but instead was dependent on IL-13. There is considerable debate over the necessity for eosinophils in the pathogenesis of airway reactivity (43–45). Although some studies in mice have shown that IL-5 is essential for the development of AHR (16–19), others have not (21, 22, 46). In our experiments, we found that the WT OVA-*indo* group had significantly higher AHR compared with the WT OVA, WT mock-*indo*, and WT mock groups. There was a small increase in the AHR of the WT OVA group compared with the WT mock group, but this increase was statistically insignificant. This diminished AHR response in the WT OVA group compared with the nonsensitized control may reflect a strain influence on the development of AHR, as C57BL/6 mice have diminished airway reactivity as compared with BALB/c mice (47, 48). The IL-5 KO OVA-*indo* group had significantly greater airway reactivity compared with the IL-5 KO OVA group and the two IL-5 KO nonsensitized groups, and these three groups were not statistically significantly different from each other. These results show that COX inhibition during allergic sensitization has profound effects on airway physiology in WT and IL-5 KO mice that are on a C57BL/6 background. Our results further show that IL-13 is responsible for the increased AHR in both the WT and IL-5 KO OVA-*indo* groups, as lung expression

of IL-13 was significantly increased in these two groups compared with their respective OVA groups and IL-13 KO OVA-*indo* mice did not have significantly greater AHR compared with either IL-13 KO OVA mice or either of the two nonsensitized groups.

These findings are consistent with several studies examining the role of IL-13 on airway inflammation and pulmonary physiology. In a mouse model, anti-IL-13 Ab treatment significantly reduced AHR while having no effect on eosinophilia (49). In this study, combined administration of anti-IL-5 and anti-eotaxin Abs before allergen challenge inhibited airway eosinophilia, but did not alter AHR (49). Intratracheal administration of rIL-13 induced AHR from 6 to 24 h after administration, and this increase in AHR was dissociated from airway eosinophilia. These investigators also found that the IL-13-mediated increase in AHR was independent of IL-5 and eotaxin (50). Additionally, adoptive transfer of Th2 polarized cells from IL-13 KO mice that make high levels of IL-4 and IL-5 led to airway inflammation, yet failed to induce AHR (51), and this induction of AHR was dependent on signaling through IL-4R α (52). Taken together, these studies show that IL-13 critically regulates AHR, without much effect on airway inflammation.

In summary, we found that LT are not necessary for the increase in the allergen-induced inflammatory response and AHR that results from COX inhibition during the development of allergic inflammation. We also found that the increase in airway eosinophilia seen with COX inhibition is dependent on IL-5 whereas the increase in AHR is not dependent on this cytokine. In contrast, the COX inhibition-mediated increase in AHR is dependent on IL-13, but airway eosinophilia is not. These results confirm that COX products are important regulators of allergic inflammation.

Disclosures

The authors have no financial conflict of interest.

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