

Cyclooxygenase Inhibition during Allergic Sensitization Increases STAT6-Independent Primary and Memory Th2 Responses¹

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Immune sensitization and memory generation are required for the development of allergic inflammation. Our previous studies demonstrate that the cyclooxygenase (COX) metabolic pathway is actively involved in allergic responses and COX inhibition increases allergic airway inflammation in a STAT6-independent fashion. To test the hypothesis that COX inhibition augments allergic inflammation by enhancing immune sensitization and memory, we sensitized STAT6 knockout mice with an i.p. injection of OVA with aluminum hydroxide as an adjuvant and treated the mice with the COX inhibitor indomethacin or vehicle for analyses of the primary and memory immune responses. We found that COX inhibition during immune sensitization, but not the allergic challenge phase, was necessary and sufficient to increase allergic inflammation. COX inhibition during sensitization increased the numbers of mature dendritic cells and activated CD4 T cells in the spleen and augmented OVA-specific IL-5 and IL-13 responses of the splenic CD4 T cells at day 5 after sensitization. COX inhibition during sensitization also augmented allergic Th2 response to OVA challenge 90 days after the sensitization. Therefore, COX inhibition during allergic sensitization augments allergic responses by enhancing Th2 cell activation and memory generation and the proallergic effect is STAT6-independent. These findings provide a mechanistic explanation for the increased allergic inflammation previously shown in the mice treated with COX inhibitors and in COX-deficient mice and suggest that use of COX-inhibiting drugs during initial allergen exposure may increase the risk of developing allergic responses. *The Journal of Immunology*, 2008, 181: 5360–5367.

Immune sensitization and memory generation are essential steps for the development of allergy, an immune disorder mediated by Th2 responses. It is difficult to identify and study the process of allergic sensitization in the clinical setting because allergic sensitization and immune memory generation are asymptomatic events of the immune response. Consequently, the mechanism by which the immune system is primed for a Th2-skewed allergic disorder is not well understood. As the prevalence of allergic diseases has doubled in the last four decades (1, 2), there is a critical need to determine the environmental factors that affect the development of allergic disorders. Recent epidemiological studies indicated that frequent use of cyclooxygenase (COX)³-inhibiting drugs was associated with increased risk of developing allergic disorders and asthma (3). The COX metabolic pathway

appears to be actively involved in the development and manifestation of allergic disorders.

COX enzymatic activities lead to the production of downstream lipid products including PGD₂, PGE₂, PGF_{2α}, PGI₂, and thromboxane A₂ in the arachidonic acid metabolic pathway. There are at least two COX isoenzymes. COX-1 is constitutively expressed in most types of cells and results in the formation of PGs with multiple physiological functions such as regulating cardiovascular tone and protecting the stomach and kidney from damage (4). COX-2 is in general induced by inflammatory stimuli and generates lipid mediators that regulate inflammatory and immune responses (4). Studies with animal models of allergic inflammation indicate that either deficiency of COX enzymes or COX inhibition with chemical inhibitors augments allergic inflammation, airway hyperresponsiveness, and Th2 immune responses. For instance, inhibition of COX-1 and/or COX-2 by nonselective or selective COX inhibitors in mice enhanced allergic inflammation as indicated by increased lung eosinophilia, Th2 cytokine production, serum allergen-specific IgE levels, and airway hyperreactivity (5–8). COX-1 deficiency in mice exhibited an enhanced allergic response (9, 10), whereas COX-2 deficiency resulted in either enhanced (5, 9, 11) or unchanged (10) allergic responses. These studies suggest that the overall effect of COX products in allergic responses is immune suppressive and COX inhibition has proallergic effects.

Newly activated CD4 T cells differentiate into the Th2 cell subset after exposure to IL-4 during the development of allergic inflammation (12). Binding of IL-4 to IL-4Rα receptor leads to a rapid phosphorylation of STAT6 that in turn induces GATA3 expression and subsequent production of the Th2 cytokines IL-4, IL-5, and IL-13 (13). In our previous study, we found that COX inhibition with indomethacin increased OVA-induced pulmonary

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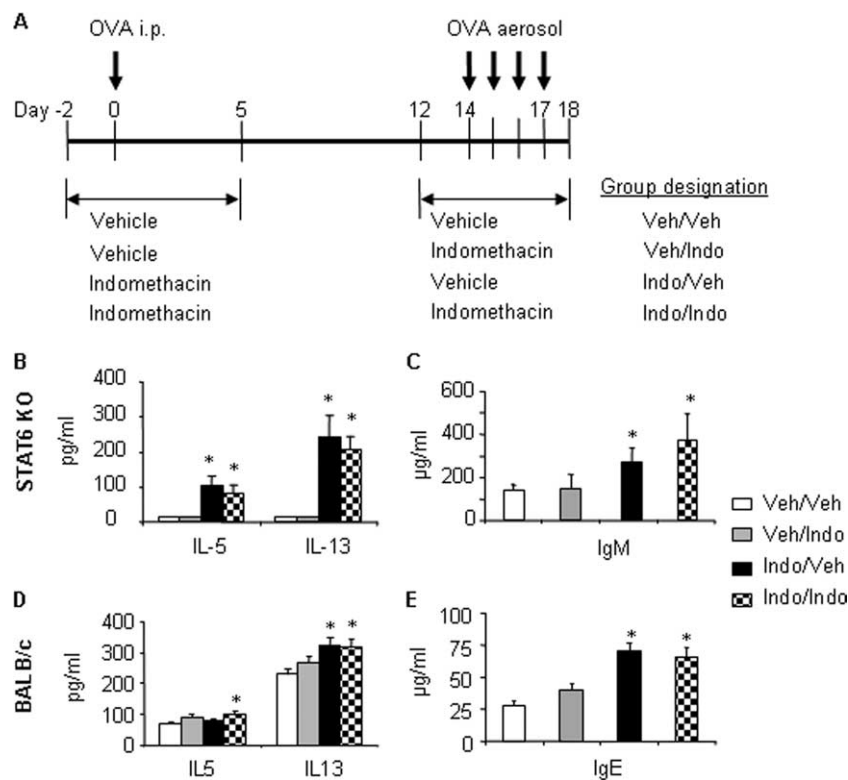
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³ Abbreviations used in this paper: COX, cyclooxygenase; KO, knockout; BAL, bronchoalveolar lavage; DC, dendritic cell; alum, aluminum hydroxide.

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FIGURE 1. COX inhibition during immune sensitization is necessary and sufficient for increasing Th2 responses to OVA challenge in STAT6 KO mice. **A**, Protocols for indomethacin treatments and for OVA sensitization and challenge. STAT6 KO (**B** and **C**) and BALB/c mice (**D** and **E**) were sensitized with an i.p. injection of OVA/alum and treated with indomethacin (30 μ g/ml) or vehicle (1% ethanol) in drinking water during either sensitization (day -2 to 5) or challenge phase (days 12–18), or both, followed by challenge with daily OVA aerosols from day 14 to 17. At day 18, mouse lungs were harvested. The levels of IL-5 and IL-13 in the ground lung supernatant (**B** and **D**) and serum IgM (**C**) and IgE (**E**) levels were measured by ELISA. *, $p < 0.05$, vs Veh/Veh group (ANOVA and Bonferroni's post test). The data (mean $\pm$ SEM) are a combination of two experiments ($n = 9\text{--}11$ per group).



IL-5 and IL-13 responses in a mouse model of OVA-induced allergic airway inflammation, and the stimulatory effect of COX inhibition was independent of the IL-4R α /STAT6-mediated signaling pathway (8). Since STAT6-mediated signaling is the classical pathway driving Th2 cell differentiation, our finding suggests that a previously described STAT6-independent nonclassical Th2 differentiation (14–17) is strongly activated by COX inhibition.

The mechanism by which COX inhibition augments STAT6-independent Th2 immune response and allergic inflammation is unknown. In this study, we tested the hypothesis that COX inhibition increases STAT6-independent immune sensitization and memory responses in mice. We found that COX inhibition during the sensitization, but not the allergic challenge phase, is necessary and sufficient for the proallergic effect of COX inhibition. We further demonstrated that COX-inhibition during the sensitization phase resulted in augmented allergic Th2 responses 90 days after sensitization, revealing the lasting impact of COX inhibition on the immune memory.

Materials and Methods

Mice

BALB/c mice and STAT6 knockout (KO) mice on a BALB/c background were purchased from The Jackson Laboratory. Female mice were age-matched and used between 8 and 12 wk of age. 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 (revised 1996).

Allergic sensitization, challenge, and COX inhibition

As shown in Fig. 1A, wild-type BALB/c and STAT6 KO mice were OVA sensitized by an i.p. injection of 0.1 ml OVA solution (10 μ g OVA formulated with 20 mg of aluminum hydroxide (alum)) at day 0, followed by daily exposure to aerosols of 1% OVA solution in PBS using an ultrasonic nebulizer for 40 min from day 14 to 17. The mice were treated with the COX inhibitor indomethacin or vehicle during sensitization from day -2 to 5 (starting 2 days before the immunization and continuing until 5 days

after the immunization), or during challenge from day 12 to 18, or both. Indomethacin (30 μ g/ml) or vehicle (1% ethanol) was administered in the drinking water. At day 18, the lungs were harvested for cytokine measurements. Mouse sera were prepared for determination of the levels of IgM, IgG1, IgG2a, IgG2b, IgA, and IgE by using the SBA Clonotyping System/HRP kit (Southern Biotechnology Associates) according to the manufacturer's instructions. Bronchoalveolar lavage (BAL) fluid was collected for total and leukocyte differential cell counts (8). In some experiments, OVA-immunized mice were treated with indomethacin or vehicle from day -2 to 5, mouse spleens and/or Peyer's patches were harvested at days 2, 5, 30, and 90 for analysis of Th2 cytokine responses. In long-term challenge experiments, the mice were OVA/alum sensitized at day 0 and treated with indomethacin or vehicle in drinking water during the sensitization phase from day -2 to 5. After day 5, normal drinking water was given to the mice. The mice were challenged by daily exposure to aerosols of 1% OVA solution starting either from day 30 to 33 or from day 90 to 93. Mouse lungs were harvested at 1 day after the last aerosol at days 34 or 94 for cytokine measurements and cell culture.

Cell preparation and culture

Single-cell suspension of the lung, spleen, and Peyer's patches were prepared by grinding the tissues through a nylon cell strainer (70 μ m; BD Biosciences) with 3 ml of RPMI 1640 medium. The mononuclear cells in the lung were purified by centrifugation on Fico/Lite-LM (density at 1.086 g/L; Atlanta Biologicals) according to the manufacturer's instructions. For spleen cells, lysis of RBC was performed with a hypotonic ammonium chloride solution (BioLegend). CD4 cells in the spleen and Peyer's patches were purified by using Miltenyi Biotec anti-CD4 microbeads. The RBC-depleted spleen cells, purified splenic CD4 cells, and CD4 cells of Peyer's patches were resuspended at 5×10^6 , 2×10^6 , and 2×10^6 cells/ml, respectively, and cultured in RPMI 1640 complete medium (Mediatech) supplemented with 10% FBS (HyClone), 4 mM L-glutamine, 1 mM sodium pyruvate, 55 μ M β -ME, 10 mM HEPES, 100 U/ml penicillin, and 100 μ g/ml streptomycin in 96-well plates. These cells were stimulated with OVA protein (100 μ g/ml), BSA (100 μ g/ml), or PMA (1 ng/ml) and ionomycin (1 μ g/ml) for 3 days. The culture supernatant was harvested at day 3 for cytokine measurements.

Cytokine, chemokine, and PGE₂ measurement

Cytokines (IL-5 and IL-13) in the ground lung supernatant, ground spleen supernatant, cell culture supernatant, and chemokines (CCL21, CXCL13, KC, and TARC) in the ground spleen supernatant were assayed by R&D

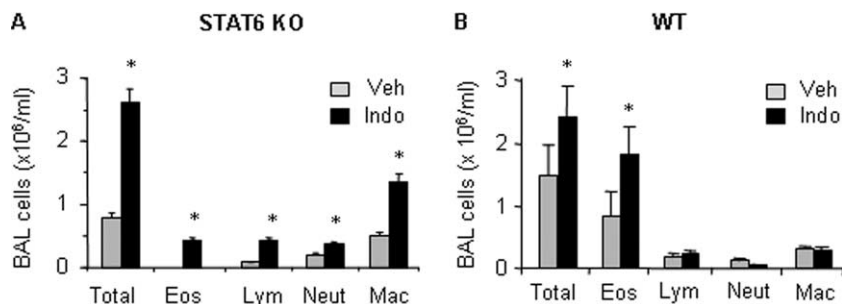


FIGURE 2. COX inhibition during sensitization increases inflammatory cell infiltration in the lung after OVA challenge. STAT6 KO (A) and BALB/c mice (B) were OVA sensitized and treated with indomethacin or vehicle from day -2 to 5 , and challenged with daily OVA aerosols from day 14 to 17 . At day 18 , BAL fluid was collected for total and differential cell counts for eosinophils, lymphocytes, macrophages, and neutrophils. *, $p < 0.05$, vs Veh (Student's t test). The data shown (mean $\pm$ SEM) are a combination of two experiments ($n = 9$ per group).

Quantikine and DuoSet ELISA kits (R&D Systems). PGE₂ in the ground spleen supernatant was determined by an R&D Parameter ELISA kit.

Flow cytometry

The lung mononuclear cells were stimulated *in vitro* with PMA (1 ng/ml) and ionomycin (1 μ g/ml) in the presence of GolgiStop (BD Biosciences) at 0.7 μ g/ml to block cytokine secretion for 6 h. The splenic cells were cultured and stimulated with OVA for 3 days and GolgiStop was added in the last 6 h of the culture. The cells were stained with fluorochrome-labeled Abs against CD4, CD8, B220, IL-5, and IL-13 with an intracellular cytokine staining protocol (BD Pharmingen). In some experiments, freshly prepared nucleated cells of the spleen were stained with fluorochrome-labeled Abs against CD11c, CD11b, CD86, MHC II, CD40, CD4, and CD44. Cells were analyzed by flow cytometry with a LSR II flow cytometer (BD Biosciences).

Statistics

The p values were calculated by using ANOVA with Bonferroni's post test for multiple group comparisons and the unpaired Student t test for two group comparisons. Values of $p < 0.05$ were considered significant.

Results

COX inhibition during sensitization is necessary and sufficient for increasing STAT6-independent allergic response

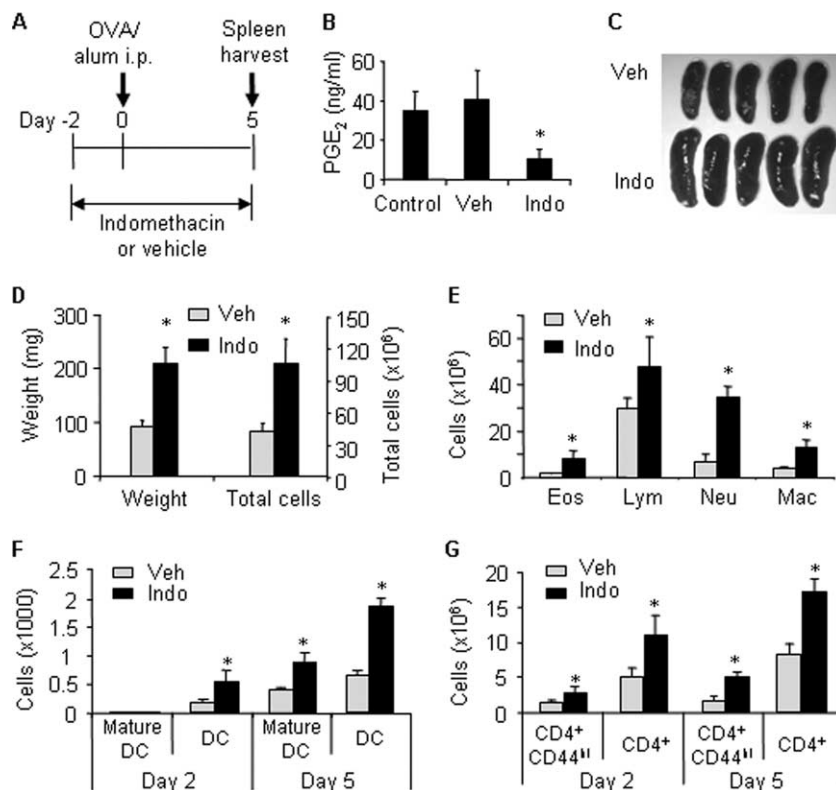
In our published work, we showed that COX inhibition during the development of OVA-induced airway inflammation (a period encompassing allergic sensitization and challenge phases) increased the allergic airway responses to OVA challenge in a STAT6-independent manner (8). To determine whether COX inhibition during sensitization and/or challenge phase was required for the increased allergic inflammation, we used a protocol in which mice were treated with the COX inhibitor indomethacin or vehicle during either OVA sensitization or OVA challenge, or both phases (Fig. 1A). To focus on the STAT6-independent effect of COX inhibition on allergic inflammation, we used STAT6 KO mice for most of the experiments in this study. We found that STAT6 KO mice treated with indomethacin during sensitization alone (Fig. 1B, Indo/Veh group) had significantly increased production of IL-5 and IL-13 in the ground lung supernatant after OVA challenge, compared with the vehicle-treated mice (Fig. 1B, Veh/Veh group). The magnitude of the stimulatory effect of COX inhibition during sensitization (Indo/Veh group) is comparable with that of COX inhibition during both sensitization and challenge phases (Indo/Indo group), as indicated by similar levels of IL-5 and IL-13 in the lung (Fig. 1B). In contrast, mice treated with indomethacin during the challenge phase alone (Veh/Indo group) did not have a detectable level of IL-5 and IL-13 (Fig. 1B), which was identical with vehicle-treated mice (Fig. 1B, Veh/Veh group). Treatment of mice with indomethacin during sensitization, but not the challenge phase, also increased the level of total serum IgM compared with

vehicle controls (Fig. 1C), suggesting that COX inhibition during sensitization increases the primary humoral immune response. STAT6 KO mice did not develop an IgE response (data not shown), which is consistent with our previous report (8). Indomethacin treatment of mice during sensitization, challenge, or both phases did not change the serum levels of IgG1, IgG2a, IgG2b, and IgA compared with vehicle-treated mice (data not shown). Therefore, COX inhibition during sensitization, but not during the challenge phase, is necessary and sufficient to augment the allergic Th2 cytokine response and IgM Ab response. The stimulatory effect of COX inhibition is independent of STAT6-mediated signaling pathway.

To determine the effect of COX inhibition on a genetically unmodified mouse strain, we conducted similar experiments with BALB/c mice. We found that indomethacin treatment during both sensitization and challenge phases (Indo/Indo) significantly increased levels of the IL-5, IL-13, and IgE (Fig. 1, D and E, vs Veh/Veh). Treatment of mice with indomethacin during sensitization, but not during the challenge phase, significantly augmented IL-13 production in the lung and increased serum IgE levels, compared with the vehicle controls (Fig. 1, D and E). These results suggest that COX inhibition acts on the immune sensitization phase to increase the development of allergic responses. Indomethacin treatment during sensitization alone did not significantly increase IL-5 production (Fig. 1D) probably because the IL-5 response peaks after 2 days of OVA challenge (6) in the model of OVA-induced allergic responses and we might have missed the peak at the time of mouse harvest after 4 days of OVA challenge. Because of the high baseline levels of allergic inflammation in BALB/c mice, the magnitude of the stimulatory effect of COX inhibition on wild-type allergic responses was statistically significant, but not as great as that in STAT6 KO mice.

To examine the effect of COX inhibition on the inflammatory response in the lung after OVA challenge, we treated STAT6 KO and BALB/c mice with indomethacin or vehicle from day -2 to 5 , and challenged the mice with OVA aerosols from day 14 to 17 . At day 18 , BAL fluid was collected for total and differential cell counts. STAT6 KO mice treated with indomethacin had greater numbers of total cells, eosinophils, lymphocytes, neutrophils, and macrophages in the BAL fluid compared with those treated with vehicle (Fig. 2A). Similarly, treatment of BALB/c mice with indomethacin during sensitization increased the numbers of total cells and eosinophils in the BAL fluid (Fig. 2B). Therefore, COX inhibition during sensitization augmented inflammatory cell infiltration to the airway in the allergic response and the effect is STAT6 independent.

FIGURE 3. COX inhibition during sensitization augments the number and activation status of DCs and CD4 T cells in the spleen. **A**, STAT6 KO mice were sensitized with an i.p. injection of OVA/alum and treated with indomethacin or vehicle from day -2 to 5. At day 5, mouse spleens were harvested. **B**, PGE₂ levels in the ground spleen supernatant of the control mice (with neither OVA sensitization nor treatment with indomethacin or vehicle), vehicle-treated mice (Veh), and indomethacin-treated mice (Indo) were determined by ELISA. The spleen size (**C**), weight (**D**), total nucleated cells (**D**), and the number of different types of leukocytes (**E**) in the spleen were determined. The splenic cells were also stained with fluorochrome-labeled Abs for flow cytometric analyses to determine the number of DCs (CD11c⁺CD11b⁺ cells) and mature DCs (CD11c⁺CD11b⁺CD86⁺CD40⁺MHC II^{high} cells) (**F**), and CD4 cells and activated CD4 cells (CD4⁺CD44^{high}) (**G**) in the spleen. *, $p < 0.05$, vs vehicle-treated mice (Student's t test). The data (mean $\pm$ SD of five mice per group) are one representative of 4 independent experiments.



COX inhibition during sensitization increases the size of the spleen and the numbers of mature dendritic cells (DCs) and activated CD4 T cells in the spleen

Based on the finding that the proallergic effect of COX inhibition is mediated by its impact on allergic sensitization, we hypothesized

that COX inhibition increases the primary immune responses to OVA/alum immunization. To test this hypothesis, we gave STAT6 KO mice an i.p. injection of OVA/alum at day 0 and treated the mice with indomethacin or vehicle from day -2 to 5 (Fig. 3A). We analyzed the primary immune responses at day 5 in the spleen. We

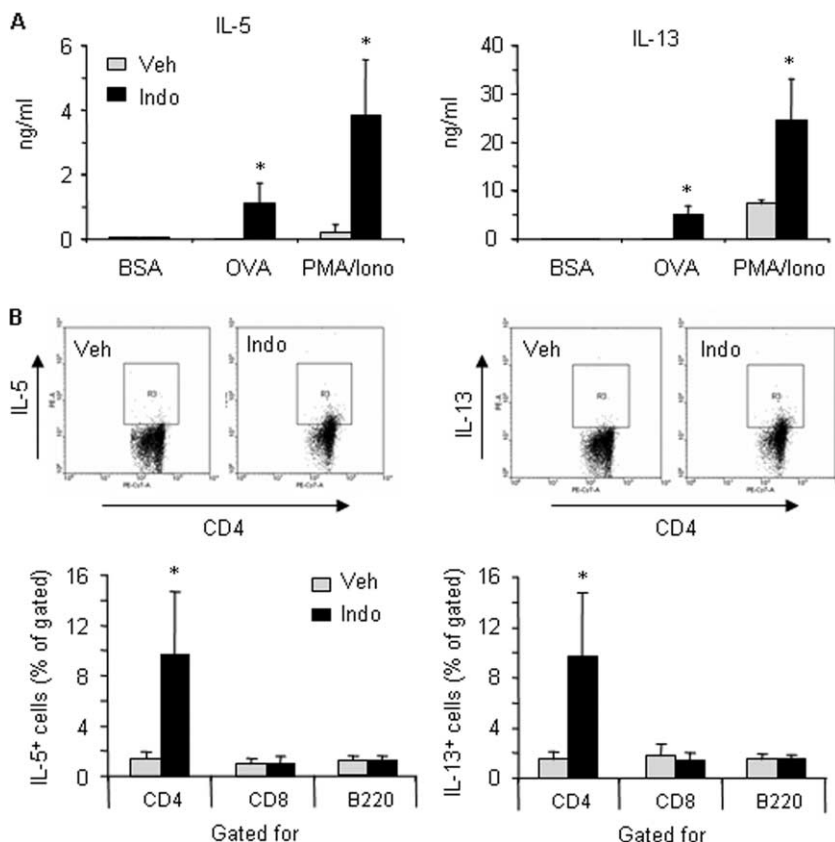
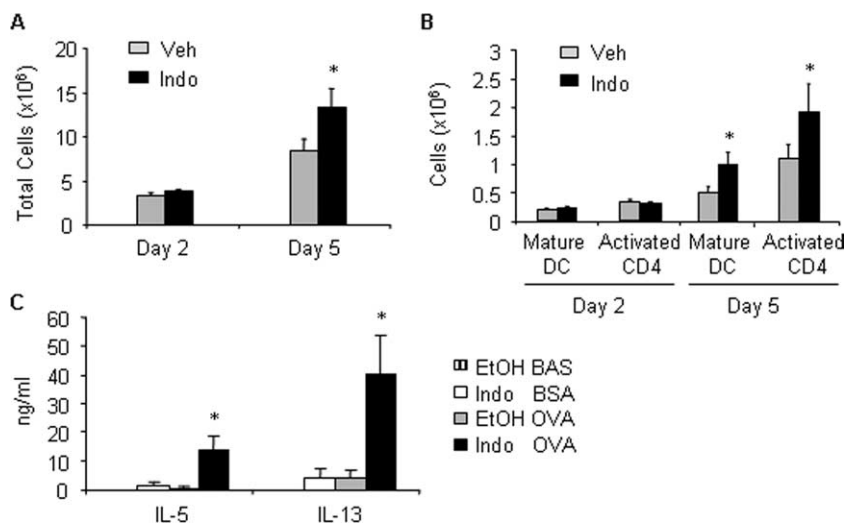


FIGURE 4. COX inhibition during sensitization increases the primary CD4 cell response. STAT6 KO mice were sensitized with an i.p. injection of OVA/alum and treated with indomethacin or vehicle from day -2 to 5. At day 5, spleens were harvested and the splenocytes were cultured in vitro and stimulated with BSA (100 μ g/ml), OVA (100 μ g/ml), or PMA (1 ng/ml) and ionomycin (1 μ g/ml) for 3 days. **A**, IL-5 and IL-13 production in the culture supernatant were assayed by ELISA. **B**, After GolgiStop was added to the media in the last 6 h of culture, the cells were surface stained for CD4, CD8, and B220, and intracellularly stained for IL-5 and IL-13 for flow cytometric analyses as gated for CD4 cells. *, $p < 0.05$, vs vehicle-treated mice (Student's t test). Data (mean $\pm$ SD of five mice per group) are one representative of four (**A**) and two (**B**) independent experiments.

FIGURE 5. COX inhibition during sensitization increases the primary immune responses in Peyer's patches. STAT6 KO mice were sensitized with an i.p. injection of OVA/alum and treated with indomethacin or vehicle from day -2 to 5. At day 2 and 5, mouse Peyer's patches were harvested. **A**, The total numbers of cells were counted. **B**, Numbers of mature DCs (CD11c⁺CD86⁺MHC II^{high} cells) and activated CD4 cells (CD4⁺CD44^{high}) were determined by flow cytometry. **C**, The cells were in vitro stimulated with OVA (100 μ g/ml) or BSA (100 μ g/ml) for 3 days. The levels of IL-5 and IL-13 in the culture supernatant were determined by ELISA. *, $p < 0.05$, vs vehicle-treated mice (Student's t test). Combined data of two experiments are shown (mean $\pm$ SEM, $n = 10$ –12 per group).



found that treatment of mice with indomethacin resulted in 4-fold reduction of PGE₂ production in the spleen (Fig. 3B), indicating an effective suppression of COX products by indomethacin. Indomethacin treatment during sensitization resulted in about 2-fold increases in the size, weight, and total number of nucleated cells in the spleen compared with vehicle-treated mice (Fig. 3, C and D). There were greater numbers of eosinophils, lymphocytes, neutrophils, and macrophages in the spleen of indomethacin-treated mice than vehicle-treated mice (Fig. 3E), suggesting an increased recruitment and/or proliferation of the inflammatory cells in the spleen. At days 2 and 5 after OVA/alum injection, the total numbers of DCs as defined as CD11c⁺CD11b⁺ cells by flow cytometric analyses and the number of mature DCs (CD11c⁺CD11b⁺CD86⁺CD40⁺MHC II^{high} cells) in the spleens of indomethacin-treated mice were approximately twice as many as those in vehicle-treated mice (Fig. 3F). Similarly, the number of CD4 cells and activated CD4 cells (CD4⁺CD44^{high}) were greater in the spleens of indomethacin-treated mice than in vehicle-treated mice (Fig. 3G). These data suggest that COX inhibition during sensitization led to increased DC influx and greater CD4 T cell activation in the spleens of STAT6 KO mice. To determine the mechanism by which COX inhibition increases the numbers of inflammatory cells in the spleens, we measured the protein levels of multiple chemokines including CCL21, CXCL13, KC, and TARC in the ground spleen supernatant. CCL21 binds to CCR7 and is expressed

in the T cell zone of lymphoid tissues for the recruitment of mature DCs and naive T cells (18). CXCL13 is a ligand for CXCR5 and is required for B cell migration into the follicles of lymph tissues (18). There was no significant difference in the levels of CCL21 and CXCL13 between the vehicle- and indomethacin-treated mice (CCL21: 2785 $\pm$ 161 pg/ml vs 3028 $\pm$ 171 pg/ml; CXCL13: 1310 $\pm$ 99 vs 1483 $\pm$ 104 pg/ml; combined data of two experiments, mean $\pm$ SEM, $n = 10$). The protein levels of KC (a neutrophil-attracting chemokine) and TARC (a Th2 cell-attracting chemokine) in the spleen were undetectable.

COX inhibition during sensitization increases STAT6-independent OVA-specific primary Th2 responses in the spleen

To further investigate the effect of COX inhibition during sensitization on the primary immune response, we prepared a single-cell suspension of nucleated spleen cells and stimulated the cells in vitro with either the immunogen OVA or pan-lymphocyte activating agents PMA and ionomycin for 3 days followed by cytokine measurement in the culture supernatant. An irrelevant protein, BSA, was used as a negative control treatment. As shown in Fig. 4A, treatment of mice with indomethacin during sensitization resulted in increased IL-5 and IL-13 production by the splenic cells in response to OVA stimulation compared with vehicle-treated mice. Stimulation of the cells of indomethacin-treated mice with BSA did not elicit a detectable IL-5 and IL-13 response, indicating

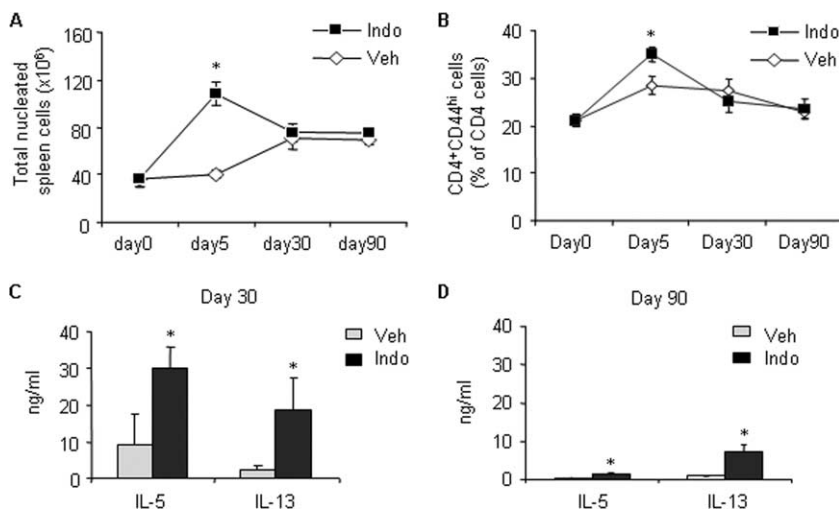
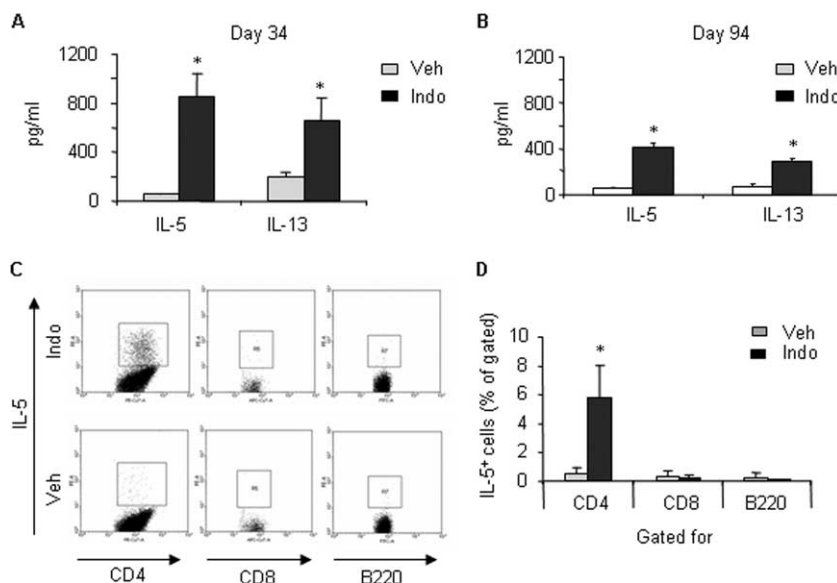


FIGURE 6. COX inhibition during sensitization increases IL-5 and IL-13 responses by spleen cells 30 or 90 days after OVA sensitization. STAT6 KO mice were sensitized with an i.p. injection of OVA/alum and treated with indomethacin or vehicle from day -2 to 5. At days 30 and 90 after OVA/alum injection, spleens were harvested for analysis of total nucleated cells by cell counting and CD4⁺CD44^{hi} cells by flow cytometry (A and B). Spleen cells were cultured and stimulated with OVA (100 μ g/ml) for 3 days. The levels of IL-5 and IL-13 in the culture supernatant were determined by ELISA (C and D). *, $p < 0.05$, vs vehicle (Student's t test). Data (mean $\pm$ SD with $n = 5$ –10 (A) or $n = 5$ (B, C, and D)) is one representative of three independent experiments (B).

FIGURE 7. COX inhibition during sensitization increases immune memory response. STAT6 KO mice were sensitized with an i.p. injection of OVA/alum and treated with indomethacin or vehicle from day -2 to 5. The mice were challenged with aerosols of OVA solution daily either from day 30 to 33 or from day 90 to 93. At days 33 and 94, respectively, the lungs were harvested. (A and B) IL-5 and IL-13 in the ground lung supernatant were assayed by ELISA. C, Single-cell suspension of lung mononuclear cells were prepared and cultured with PMA (1 ng/ml), ionomycin (1 μ g/ml), and GolgiStop (0.7 μ g/ml) for 6 h. The cells were surface stained for CD4, CD8, and B220, and intracellularly stained for IL-5 for flow cytometric analyses. *, $p < 0.05$, vs vehicle (Student's t test). Data (mean $\pm$ SD with $n = 5$ (A, B, and D)) are one representative of three (A and B) or two (C and D) independent experiments.



that the Th2 cytokine responses were OVA-specific (Fig. 4A). PMA/ionomycin stimulation resulted in 3–10-fold increases in IL-5 and IL-13 production by the cells of indomethacin-treated mice compared with vehicle controls (Fig. 4A), suggesting that COX inhibition increased the potential of Th2 cytokine production by the splenic cells. To determine the cellular source of the cytokines, we determined IL-5 and IL-13 expression by intracellular cytokine staining and flow cytometric analyses. We found that splenic CD4 cells of indomethacin-treated mice (Fig. 4B), but not CD8- or B220-positive cells, produced IL-5 and IL-13 after in vitro stimulation (Fig. 4B). The percentage of CD4 cells that produced IL-5 and IL-13 was 5-fold greater in indomethacin-treated mice than in vehicle-treated mice (Fig. 4B).

To determine whether COX inhibition also increased Th2 responses in other abdominal lymph tissues, we analyzed the effect of COX inhibition on the primary immune responses in Peyer's patches. STAT6 KO mice were given an i.p. injection of OVA/alum at day 0 and treated with indomethacin or vehicle from day -2 to 5. Mouse Peyer's patches were harvested at days 2 and 5. As shown in Fig. 5, A and B, indomethacin treatment during sensitization increased the numbers of total cells, activated ($CD4^+CD44^{\text{high}}$) CD4 cells, and mature ($CD11c^+CD86^+MHC\ II^{\text{high}}$) DCs in Peyer's patches at day 5 after OVA/alum injection. When the cells of Peyer's patches harvested at day 5 after OVA immunization were in vitro stimulated with OVA, there were greater levels of IL-5 and IL-13 in the culture supernatant of the cells from indomethacin-treated mice than from vehicle-treated mice (Fig. 5C). Stimulation of the cells with BSA resulted in minimum or undetectable levels of these cytokines (Fig. 5C), suggesting that the Th2 cytokine response is OVA-specific. In contrast, indomethacin treatment did not change the numbers of total cells, mature DCs, and activated CD4 T cells in the Peyer's patches at day 2 after OVA injection (Fig. 5, A and B). The cells of Peyer's patches that were harvested at day 2 did not produce a detectable level of IL-5 and IL-13 after in vitro stimulation with OVA. Therefore, COX inhibition during sensitization increased the primary Th2 responses not only in the spleen, but also in the Peyer's patches at day 5 after OVA immunization.

COX inhibition during sensitization increases STAT6-independent Th2 memory response

Based on the findings that COX inhibition during sensitization increases the primary CD4 T cell response in the spleen, we hy-

pothesized that COX inhibition during sensitization enhances immune memory response. To test this hypothesis, we monitored dynamic changes of total cells and activated CD4 T cells in the spleen of STAT6 KO mice for 90 days. As shown in Fig. 6, COX inhibition with indomethacin during sensitization resulted in greater numbers of not only total nucleated cells, but also activated CD4 cells in the spleen at day 5 after OVA/alum injection, compared with vehicle treatment (Fig. 6). In contrast, there was no difference in the numbers of total nucleated cells and activated CD4 T cells in the spleen between indomethacin- and vehicle-treated mice at days 30 and 90. The total number of activated CD4 cells peaked at day 5 and decreased thereafter over a period of 90 days (Fig. 6B). These data suggest a decreased immune response in the spleen of indomethacin-treated mice at days 30 and 90 after OVA/alum injection compared with that at day 5. However, when splenic cells were harvested from indomethacin-treated STAT6 KO mice at days 30 and 90 after OVA/alum injection and stimulated in vitro with OVA, they produced 3–8-fold greater levels of IL-5 and IL-13 in the culture supernatant than the cells of vehicle-treated mice (Fig. 6, C and D). When STAT6 KO mice were challenged with daily OVA aerosols either from day 30 to 33 or from day 90 to 93, treatment of the mice with indomethacin resulted in 3–20-fold greater Th2 cytokine responses in the lung, compared with vehicle treatment (Fig. 7, A and B). Flow cytometric analyses of the lung mononuclear cells indicated that CD4 cells, but not CD8 or B220 cells, produced IL-5 (Fig. 7, C and D). Therefore, COX inhibition during immune sensitization increased STAT6-independent Th2 immune memory responses to airway OVA challenge and the effect of COX inhibition prolonged for 90 days after immune sensitization.

Discussion

We have shown that COX inhibition during allergic sensitization has a strong stimulatory effect on the primary and memory immune responses in a STAT6-independent manner. In contrast, COX inhibition during the phase of allergic challenge did not affect the OVA-induced Th2 response. Therefore, COX inhibition augments the allergic responses by enhancing allergic sensitization but not by increasing the effector response of the immune cells to allergen re-exposure. Treatment of mice with indomethacin during sensitization not only increased the priming of CD4 T cells, but also augmented the allergic memory response 90 days later to OVA challenge.

Our results indicate that COX inhibition during sensitization is necessary and sufficient for augmenting the OVA/alum-induced immune primary and memory responses. We found that COX inhibition during either the peri-sensitization phase from day -2 to 5 or during a shorter period of time from day -2 to 2 (data not shown) increased allergic responses to OVA challenge. Consistently, Schleimer and Benjamini (19) demonstrated that mice immunized through an i.p. injection of chicken serum albumin in PBS and treated with either the COX inhibitor indomethacin or RO 20-5720 during the sensitization (24 h before, 5 min before, and 24 h after the immunization) had an increased Ab response specific for chicken serum albumin. Inoue et al. (20) reported that COX-2 inhibition with epicutaneous application of NS398 onto tape-stripped mouse skin 15 min before exposure to OVA/CpG-oligodeoxynucleotide resulted in increased immune response. Therefore, COX inhibition in a narrow time window during the initial Ag exposure appears to be necessary and sufficient for up-regulation of immune sensitization. In contrast, COX inhibition during allergic challenge phase did not change the magnitude of respiratory allergic response to OVA challenge. It is possible that COX pathway is not a determining factor for the elicitation of allergic response, though production of the COX product PGE_2 was up-regulated by OVA challenge (7).

The strong stimulatory effect of COX inhibition on the primary immune response suggests that COX products play an important role in regulating the initiation of the immune response. COX inhibition during immune sensitization increased total numbers of DCs and mature DCs in the spleen (Fig. 3), and possibly also enhanced their T cell stimulatory function. This possibility is supported by the inhibitory effect of individual COX products including PGE_2 and PGI_2 on DC maturation, inflammatory cytokine, and chemokine production, and their ability to stimulate and activate T cells (21–23). PGE_2 and PGI_2 may exert their T cell suppressive effect not only indirectly by inhibiting DCs, but also directly by decreasing T cell activation and effector cytokine production (24–26). We found high basal levels of PGE_2 in the spleen of normal mice, suggesting that PGE_2 may limit the immune responses by its direct suppressive functions on T cells. By inhibiting the production of PGE_2 and PGI_2 , COX inhibition may attenuate the indirect and direct immune suppressive effect of these PGs on T cell activation and therefore enhances the development of allergic responses.

COX inhibition during sensitization increased IL-5 and IL-13 production in the lung when the sensitized mice were OVA challenged 2 wk, 1 mo, or 3 mo after sensitization. Our data indicate that CD4 T cells are a major source of IL-5 in the lung (Fig. 7) (8), though eosinophils and mast cell are capable of producing IL-5 (27, 28). CD4 T cells play an essential role in the allergic responses in the mouse model of OVA-induced allergic airway inflammation. We have previously shown that depletion of CD4 T cells during allergic challenge totally abrogates allergic inflammation and Th2 cytokine production in the lung (8). Therefore, activation of CD4 T cells and the effector cytokine production by OVA-specific Th2 CD4 T cells precedes the infiltration of eosinophils during the allergic response. Although IL-5 produced by CD4 Th2 cells initiates eosinophil proliferation and triggers the recruitment of eosinophils to the lung, IL-5 and other factors expressed by eosinophils may further enhance eosinophilia and in turn augments IL-13 production by CD4 T cells (29). IL-13 produced by CD4 T cells stimulates mucous hypersecretion and mediates airway hyperreactivity, one of the hallmarks of allergic responses. COX inhibition during sensitization results in a greater Th2 response to in vitro OVA stimulation by the cells in the spleen

and augmented allergic Th2 memory responses in vivo at 1 and 3 mo after sensitization.

Indomethacin treatment of mice resulted in splenomegaly with increased numbers of inflammatory cells including lymphocytes, eosinophils, neutrophils, and macrophages in the spleen of STAT6 KO mice. These data suggest that treatment of the mice with indomethacin disturbs cellular homeostasis in the spleen. Although COX inhibition clearly augmented DC maturation and CD4 T cell responses in the spleen, it is still to be determined whether eosinophils, neutrophils, macrophages, and B cells also contributed to the T cell activation and initiation of the immune response. We did not find differences in CCL21 and CXCL13 protein expression in the spleens of mice treated with indomethacin or vehicle, suggesting that COX inhibition may not increase the cellularity by up-regulating these specific chemokines. Therefore, the mechanism by which COX inhibition leads to increased mature DCs, neutrophils, eosinophils, and lymphocytes in the spleen is not yet determined.

COX inhibition with indomethacin during sensitization increased OVA-specific IL-5 and IL-13 responses of the cells from the spleen and Peyer's patches at day 5 after OVA immunization. These data indicate a role of these two lymph tissues in the proallergic effect of COX inhibition. However, it is still to be determined whether the spleen and Peyer's patches are the draining lymph tissues for the peritoneal cavity, although the influx of mature DCs and T cell activation in these lymph tissues suggests that they are. Recently, Kool and colleagues (30) reported that the mediastinal lymph nodes are a draining lymph tissue for the peritoneum and demonstrated the presence of mature OVA-bearing $\text{CD11c}^+\text{CD86}^+\text{MHC II}^+$ cells in the mediastinal lymph nodes at as early as 24 h after an i.p. injection of OVA. Nevertheless, treatment of mice with the COX inhibitor indomethacin augmented the Th2 responses both in the spleen and in the Peyer's patches suggests that the effect of COX inhibition is not limited to a specific lymph tissue.

The proallergic and Th2-stimulatory functions of COX inhibition suggest that the overall effect of COX metabolic pathway is immune-suppressive. This is supported by studies showing increased allergic responses in COX-1 KO, COX-2 KO, IP KO, and EP3 KO mice (5, 9–11, 31–33), and in mice that were treated with nonselective or selective COX inhibitors (5–8, 20). COX inhibition or deficiency increased allergic inflammation not only in mice on a BALB/c genetic background (5, 8, 19) but also in mice on C57BL/6, 129, and A/J backgrounds (19, 20, 32–34), suggesting that the COX inhibition-mediated effect is not mouse strain dependent. In addition, COX inhibition augmented Th2 responses in mice that were immunized with an i.p. injection of OVA formulated with alum as an adjuvant (8). The stimulatory effect of COX inhibition on allergic responses was also reported in the mice that were immunized through epicutaneous OVA exposure without an adjuvant (5). Furthermore, selective COX-2 inhibition with NS398 augmented a Th1 immune response in mice that were epicutaneously immunized with OVA plus CpG-oligodeoxynucleotide (20). These data suggest that augmentation of immune responses by COX inhibition is not dependent on the type of immune responses, nor on mouse strain, sensitization route, and the use of adjuvant. We are actively pursuing determining the specific prostanoid, which when inhibited, leads to STAT6-independent augmentation of immune responses.

In summary, COX inhibition during immune sensitization, but not the allergic challenge phase, is necessary and sufficient for augmentation of allergic Th2 responses in a STAT6-independent fashion. Our data provide a mechanistic explanation for the increased allergic inflammation previously shown in COX-deficient

mice (5, 9–11) and in the mice treated with COX inhibitors (5–8, 20). The STAT6-independent effect of COX inhibition suggests an alternative Th2 differentiation pathway that may be compensatory to the classical Th2 response and contributes to the development of allergic response. Clinically, our results suggest that COX inhibition during the initial exposure to allergens may increase the risk of development of allergic disorders, a possibility supported by recent epidemiological studies in humans (3). The strong stimulatory effect of COX inhibition on immune sensitization and memory generation may be useful not only for development of strategies to intervene allergy development but also for other biomedical fields such as vaccine studies and cancer immunology.

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Disclosures

The authors have no financial conflict of interest.

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