Cyclooxygenase-2 Regulates Th17 Cell Differentiation during Allergic Lung Inflammation

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***Rationale*: Th17 cells comprise a distinct lineage of proinflammatory T helper cells that are major contributors to allergic responses. It is unknown whether cyclooxygenase (COX)-derived eicosanoids regulate Th17 cells during allergic lung inflammation.**

***Objectives*: To determine the role of COX metabolites in regulating Th17 cell differentiation and function during allergic lung inflammation.**

***Methods*: COX-1$^{-/-}$, COX-2$^{-/-}$, and wild-type mice were studied in an *in vivo* model of ovalbumin-induced allergic inflammation and an *in vitro* model of Th17 differentiation using flow cytometry, cytokine assays, confocal microscopy, real-time polymerase chain reaction, and immunoblotting. In addition, the role of specific eicosanoids and their receptors was examined using synthetic prostaglandins (PGs), selective inhibitors, and siRNA knockdown.**

***Measurements and Main Results*: Th17 cell differentiation in lung, lymph nodes, and bronchoalveolar lavage fluid was significantly lower in COX-2$^{-/-}$ mice after ovalbumin sensitization and exposure *in vivo*. *In vitro* studies revealed significantly impaired Th17 cell differentiation of COX-2$^{-/-}$ naive CD4^{+} T cells with decreased Stat3 phosphorylation and RORγt expression. Synthetic PGF$_{2\alpha}$ and PGI$_2$ enhanced Th17 cell differentiation of COX-2$^{-/-}$ CD4^{+} T cells *in vitro*. The selective COX-2 inhibitor, NS-398, and PGF$_{2\alpha}$ receptor and PGI$_2$ receptor siRNA knockdown significantly decreased Th17 cell differentiation *in vitro*. Administration of synthetic PGs restored accumulation of Th17 cells in lungs of allergic COX-2$^{-/-}$ mice *in vivo*.**

***Conclusions*: COX-2 is a critical regulator of Th17 cell differentiation during allergic lung inflammation via autocrine signaling of PGI$_2$ and PGF$_{2\alpha}$ through their respective cell surface receptors.**

Keywords: Th17 cell; COX-2; asthma; prostaglandins; IL-17

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Cyclooxygenase (COX) enzymes are known to be important regulators of Th1–Th2 balance in allergic lung disease; however, it is not known whether COX-1– or COX-2–derived eicosanoids regulate Th17 cell function, or the mechanisms involved.

What This Study Adds to the Field

This study identifies COX-2 as a key regulator of Th17 cell differentiation and function in allergic lung inflammation via an autocrine loop that involves prostaglandin I_2, prostaglandin $F_{2\alpha}$, and their respective cell surface receptors.

CD4^{+} T cells play an important role in the initiation of the immune response by providing help to other cells and by taking on a variety of effector functions during the immune response. On antigenic stimulation, naive CD4^{+} T cells activate, expand, and differentiate into various effector subsets that are characterized by distinct functions and cytokine profiles (1). Th17 cells are a recently discovered CD4^{+} helper T-cell subset characterized by the production of IL-17A, IL-17F, and IL-22 (2). Although Th1 and Th2 cell differentiation depend on single effector cytokines (IL-12 and IL-4, respectively), Th17 differentiation is induced by the combined activity of transforming growth factor (TGF)-β and IL-6 in mice, or TGF-β and IL-21 (naive T cells) or IL-1β (memory T cells) in humans (3). In both species, these cytokines affect IL-17 production by activating and inducing the expression of key lineage-specific transcription factors, such as Stat3 and the orphan nuclear receptor RORγt (4). IL-17A plays important roles in immune responses, such as delayed-type hypersensitivity, contact hypersensitivity, and allergic airway inflammation (5). IL-17A promotes inflammation by inducing various proinflammatory cytokines and chemokines, recruiting neutrophils, enhancing antibody production, and activating T cells (6). It was previously reported that IL-17A is up-regulated in asthma and nasal polyposis, and in the latter condition its expression is resistant to topical steroids (7, 8).

Cyclooxygenases (COXs) are responsible for the formation of prostaglandins (PGs), which are involved in regulating inflammatory responses (9, 10). The two COX isoforms, COX-1 and COX-2, are expressed at varying levels in different tissues (11). COX-1 is constitutively expressed in most mammalian tissues and cells, whereas COX-2 is inducible in macrophages and other cell types at sites of inflammation (12, 13). PGs exert their actions by binding to a family of G-protein–coupled receptors. These include the DP1 and DP2 subtypes of the PGD$_2$ receptor;

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the EP1, EP2, EP3, and EP4 subtypes of the PGE_2 receptor; the $PGF_{2\alpha}$ receptor (FP); the PGI_2 receptor (IP); and the TxA_2 receptor (TP) (14). It is known that some of these receptors are expressed during T-cell differentiation, a process that is important in regulating inflammation and immune responses (15).

Recent studies suggest that COX-1 and COX-2 may play important roles in regulating the Th1–Th2 balance in allergic and nonallergic lung diseases (16). These findings demonstrate COX-2–dependent regulation of TGF-β and IL-6, key cytokines that are produced by macrophages and involved in the differentiation of naive $CD4^+$ T cells to Th17 cells. The proinflammatory effects of PGE_2 in experimental inflammatory bowel disease (17) and collagen-induced arthritis in mice (18) are mediated through IL-17. In addition, PGE_2 favors Th17 expansion and IL-17 production through activation of dendritic cells via a paracrine mechanism that also involves up-regulation of the IL-23 and IL-1 receptors on T cells (19, 20). However, it remains unknown whether (*1*) COX-1 or COX-2 are critically involved in the Th17 cell differentiation process, (*2*) COX-derived PGs other than PGE_2 are involved in Th17 cell differentiation, and (*3*) PGs directly influence Th17 cells during differentiation independent of effects on dendritic cells. Therefore, in the present study we used COX-$1^{-/-}$ and COX-$2^{-/-}$ mice to examine whether PGs regulate Th17 cell differentiation in a model of allergic lung inflammation. Our results show that the number of Th17 cells in lung and lymph nodes was dramatically decreased in COX-$2^{-/-}$ mice, but not COX-$1^{-/-}$ mice, compared with wild-type (WT) mice after ovalbumin sensitization and exposure *in vivo*. There was also significantly impaired Th17 cell differentiation and IL-17A production in COX-$2^{-/-}$ T cells *in vitro*. COX-2 was expressed in Th17 cells and regulated dose-dependently by IL-6. PG production was increased during Th17 differentiation, a phenomenon that was largely COX-2 dependent. Importantly, synthetic $PGF_{2\alpha}$ and PGI_2 partially restored the Th17 cell differentiation defect in COX-$2^{-/-}$ cells *in vitro*, and systemic administration of PGs restored accumulation of Th17 cells in lungs of allergic COX-$2^{-/-}$ mice *in vivo*. Finally, Th17 cells expressed prostanoid receptors, and Th17 cell differentiation was significantly reduced by FP and IP receptor antagonists and siRNA knockdown. Together, these results indicate that Th17 cell differentiation during allergic lung inflammation is critically regulated by COX-2–derived $PGF_{2\alpha}$ and PGI_2 via an autocrine pathway that involves binding to FP and IP receptors on T cells.

METHODS

Additional details are provided in the online supplement.

Animals

Six- to 10-week-old male COX-$1^{-/-}$ mice, COX-$2^{-/-}$ mice, and WT control mice on a hybrid C57BL6J × 129/Ola genetic background were purchased from Taconic (Germantown, NY) (16). All animal experiments were performed according to the *NIH Guide for the Care and Use of Laboratory Animals* and approved by the National Institute of Environmental Health Sciences Animal Care and Use Committee.

Ovalbumin-induced Allergic Airway Inflammation Model

Mice were immunized with ovalbumin (20 μg emulsified in 0.2 ml of aluminum hydroxide adjuvant) or vehicle (adjuvant only) by intraperitoneal injection on Days 0 and 1. Starting on Day 14, mice were exposed to 1% ovalbumin (or saline) via nebulizer for 30 minutes per day for 4 consecutive days. Forty-eight hours after the last exposure, mice were killed for tissue collection.

Bronchoalveolar Lavage Fluid and Isolation of Lung and Spleen $CD4^+$ T Cells

Bronchoalveolar lavage fluid (BALF) was collected in 2 ml of phosphate-buffered saline. Naive or allergic $CD4^+$ T cells were isolated using kits from Miltenyi Biotec (Auburn, CA). Additional experiments used cell sorting to obtain naive $CD4^+$ $CD25^-$ $CD44^{+low}$ $CD62L^+$ T cells at greater than 99% purity. Cell cultures were maintained in RPMI 1640 with 10% fetal bovine serum. Th17 differentiation was induced as described (4).

Flow Cytometric Analysis and Intracellular Cytokine Staining

Naive $CD4^+$ T cells were differentiated with TGF-β and IL-6 for 5 days and restimulated for 4 hours with 12–0-tetradecanoyl-phorbol-13-acetate, ionomycin, and brefeldin A. Intracellular cytokine staining of fixed cells was analyzed using an LSRII flow cytometer (Becton Dickinson, San Jose, CA).

siRNA Knockdown of IP and FP Receptors

Freshly isolated naive $CD4^+$ T cells from spleens of WT mice were transfected with 20 nmol of siRNAs to the IP receptor, the FP receptor, or negative control using mouse T cell Nucleofector solution (Amaxa, Koln, Germany). Transfected cells were induced to differentiate into Th17 cells and analyzed by fluorescence-activated cell sorter on Day 5.

Analysis of Cytokine Levels

Blood, BALF, or supernatants from T-cell cultures were analyzed for cytokines using commercial kits.

Histopathology and Immunostaining of Lung Tissue

Lungs were perfusion-fixed and stained with hematoxylin and eosin. For immunstaining of lung tissue, the frozen-lung sections were fixed in methanol with 0.3% H_2O_2 at 4°C, permeabilized with Triton X-100 (0.8%), and stained with fluorescent antibodies. Adjacent sections were stained with hematoxylin and eosin.

Implantation of Osmotic Minipumps for Delivery of Synthetic PGs

PGE_2, $PGF_{2\alpha}$, and the PGI_2 analog iloprost (Cayman, Ann Arbor, MI) or vehicle (15% ethanol and sterile saline) were delivered by osmotic minipumps (Alzet, Cupertino, CA) 1 week before ovalbumin exposure.

Eicosanoid Analysis in Blood and BAL Fluid

Eicosanoid levels in BALF were analyzed by liquid chromatography–tandem mass spectrometry as previously described (21).

Statistical Analyses

Data are presented as means ± SEM. Statistical comparisons were performed by randomized-design two-way analysis of variance followed by the Newman-Keuls *post hoc* test for more than two groups, or by unpaired Student *t* test for two groups. Statistical significance was defined as P less than 0.05.

RESULTS

COX-$2^{-/-}$ Mice Have Reduced Th17 Cells during Allergic Lung Inflammation *In Vivo*

Allergic lung diseases, such as asthma, are hypothesized to result from dysregulated immune responses. A previous study has demonstrated that COX products play an important role in regulating Th1–Th2 balance in both allergic and nonallergic lung diseases (16). Recent progress in characterizing the proinflammatory IL-17 cytokine family has added an additional layer of

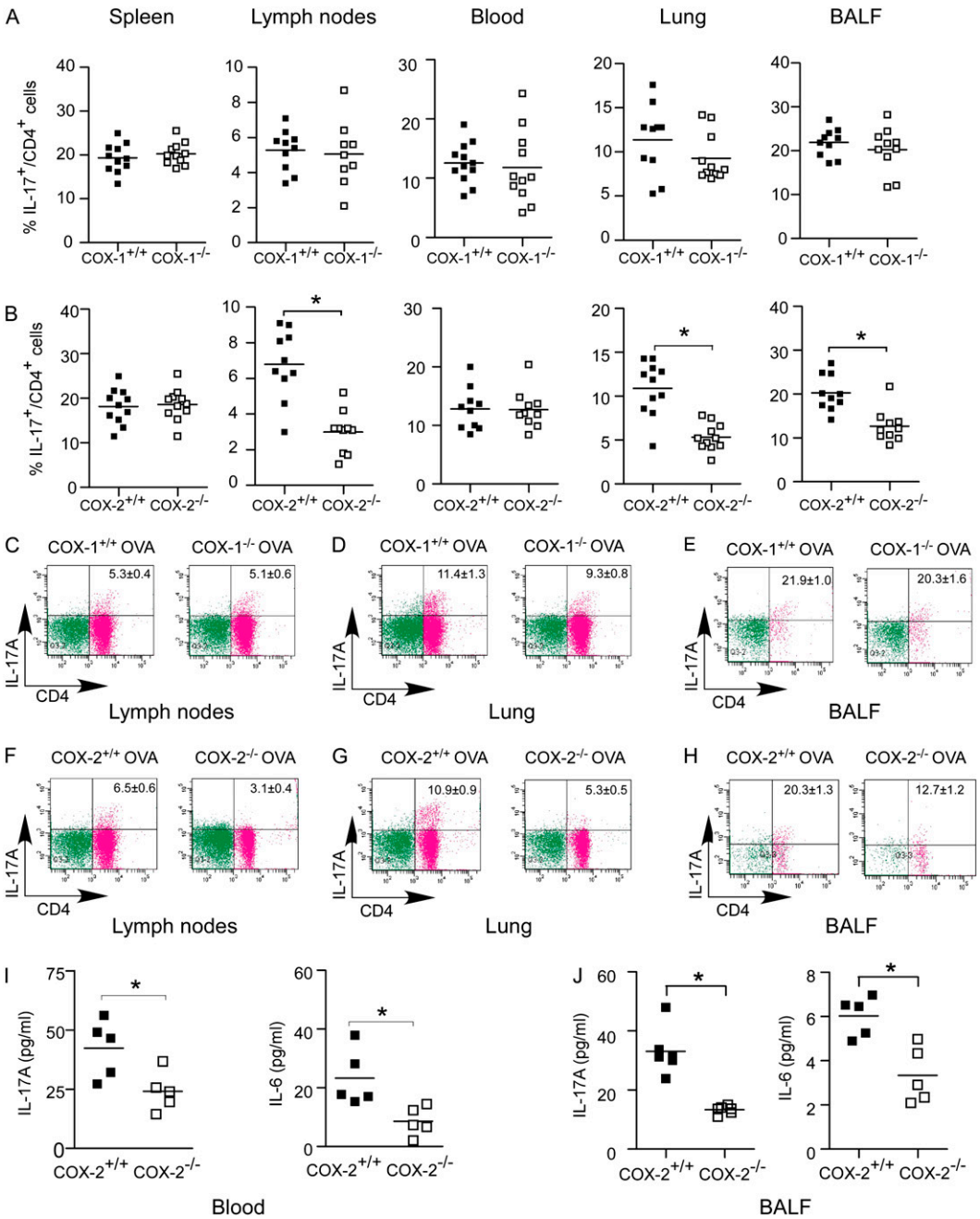

Figure 1. Reduced Th17 cells in lung, bronchoalveolar lavage fluid (BALF), and lymph nodes of cyclooxygenase (COX)-2$^{-/-}$ mice after ovalbumin (OVA) sensitization and exposure *in vivo*. COX-1$^{+/+}$, COX-1$^{-/-}$, COX-2$^{+/+}$, and COX-2$^{-/-}$ mice (n = 9–12 each) were sensitized with OVA in adjuvant. Fourteen to 21 days later, mice were exposed to inhaled OVA for 4 consecutive days. The percentages of IL-17A^{+} CD4^{+} T cells in spleen, lymph nodes, blood, lung, and BALF from COX-1$^{+/+}$ versus COX-1$^{-/-}$ mice (*A*) and COX-2$^{+/+}$ versus COX-2$^{-/-}$ mice (*B*) were analyzed by flow cytometry 48 hours after the last OVA exposure. Flow cytometry scattergrams show that the percentage of IL-17A^{+} CD4^{+} cells were similar in COX-1$^{+/+}$ versus COX-1$^{-/-}$ lymph nodes (*C*), lung (*D*), and BALF (*E*). In contrast, COX-2$^{-/-}$ mice had significantly fewer IL-17A^{+} CD4^{+} cells in lymph nodes (*F*), lung (*G*), and BALF (*H*). IL-17A and IL-6 concentrations in blood (*I*) and BALF (*J*) were measured by ELISA and BioPlex assay 48 hours after the last OVA exposure. For panels *A*, *B*, and *I*, lines indicate the mean, and each symbol (*solid squares*, COX-1$^{+/+}$ or COX-2$^{+/+}$; *open squares*, COX-1$^{-/-}$ or COX-2$^{-/-}$) represents an individual mouse. * $P < 0.05$ versus wild-type.

complexity to the understanding of the regulation of allergic lung inflammation (22). Therefore, to investigate the role of COX-1 and COX-2 in regulating Th17 cells *in vivo*, we used an established model of ovalbumin sensitization and exposure to induce allergic lung inflammation in COX-1$^{-/-}$, COX-1$^{+/+}$, COX-2$^{-/-}$, and COX-2$^{+/+}$ mice. After ovalbumin sensitization and exposure, the percentages of IL-17^{+} CD4^{+} (Th17) cells in spleen, lymph nodes, blood, lung, and BALF of COX-1$^{-/-}$ and COX-1$^{+/+}$ mice were comparable (Figures 1A and 1C–1E). In contrast, COX-2$^{-/-}$ mice showed significantly reduced percentages of Th17 cells in lung (5.3% vs. 10.9%), BALF (12.7% vs. 20.3%), and lymph nodes (3.1% vs. 6.5%), but not in spleen and blood, compared with COX-2$^{+/+}$ mice (Figures 1B and 1F–1H). The absolute number of IL-17^{+} CD4^{+} T cells in lung and BALF was also significantly decreased in COX-2$^{-/-}$ mice (*see* Figure E1 in the online supplement). Consistent with this finding, we found that IL-17A levels in blood and BALF were significantly decreased in COX-2$^{-/-}$ mice compared with COX-2$^{+/+}$ mice after ovalbumin exposure (Figures 1I and 1J). Interestingly, levels of IL-6 were also significantly decreased in blood and BALF from COX-2$^{-/-}$ mice compared with COX-2$^{+/+}$ mice after ovalbumin sensitization and exposure (Figures 1I and 1J). Together, these data suggest that Th17 cell numbers, and IL-17A and IL-6 cytokine levels, are reduced in COX-2$^{-/-}$ mice relative to WT during allergic lung inflammation *in vivo*.

To further implicate a role for COX-2 in regulating Th17 cells *in vivo*, and to exclude the contribution of compensatory pathways, such as altered levels of TGF-β, IL-6, or leukocytes in the observed phenotype, we performed an acute COX-2 inhibitor study in WT mice exposed to ovalbumin. Th17 cell percentages in lung (5.18 ± 1.13 vs. 7.25 ± 1.47; n = 6; $P < 0.01$) and blood (4.05 ± 0.59 vs. 6.07 ± 0.85; n = 6; $P < 0.01$) were significantly decreased in the COX-2 inhibitor group compared with control after ovalbumin exposure (Figure E2). Th17 cell percentages in

BALF (19.12 ± 3.66 vs. 22.42 ± 2.14; n = 6; $P > 0.05$), lymph nodes (12.03 ± 1.47 vs. 14.21 ± 0.94; n = 6; $P > 0.05$), and spleen (6.50 ± 1.17 vs. 8.40 ± 0.68; n = 6; $P > 0.05$) also tended to be lower in the COX-2 inhibitor group, but these differences did not reach statistical significance.

Localization of Th17 Cells to Sites of Allergic Lung Inflammation

Analysis of lung tissue sections stained with hematoxylin and eosin revealed that allergic COX-$2^{+/+}$ and COX-$2^{-/-}$ had increased inflammation compared with nonallergic mice that were not sensitized or exposed to ovalbumin (Figures 2A, 2E, and 2I). Consistent with our previous work demonstrating an inhibitory effect of PGs on Th2 immune responses (16, 23), lungs from allergic COX-$2^{-/-}$ mice showed increased airway inflammation compared with lungs from allergic COX-$2^{+/+}$ mice (Figures 2A and 2E). To examine the localization of Th17 cells in this model, we stained adjacent lung tissue sections with anti-CD4 and anti–IL-17A. In COX-$2^{+/+}$ mice, there were abundant Th17 cells localized to sites of allergic inflammation (Figures 2B–2D). In contrast, allergic COX-$2^{-/-}$ mice showed markedly reduced numbers of Th17 cells at sites of allergic inflammation (Figures 2F–2H). Th17 cells were not observed in nonallergic mice that were not exposed to ovalbumin (Figures 2J–2L). To quantify the number of Th17 cells *in vivo*, multiple randomly selected regions (n = 22–29) from each lung section were counted and quantified using MetaMorph software (Sunnyvale, CA). The results demonstrate the presence of significantly more lung IL-17^+/$CD4^+$ T cells in COX-$2^{+/+}$ mice than in COX-$2^{-/-}$ mice after ovalbumin exposure (Figure 2M; $P < 0.01$). Lung IL-17^+/$CD4^+$ T cells of both COX-$2^{+/+}$ and COX-$2^{-/-}$ mice were significantly increased after ovalbumin exposure compared with control (no ovalbumin exposure). Together, these data indicate that Th17 cells localize to sites of allergic lung inflammation and confirm reduced numbers of Th17 cells in inflammatory loci of COX-$2^{-/-}$ mice exposed to ovalbumin *in vivo*.

Regulation of COX-1 and COX-2 Expression during Th17 Cell Differentiation *In Vitro* and *In Vivo*

COX-2 is primarily expressed in activated monocytes and macrophages (24); however, T cells are reported to express COX-2 and produce PGE_2 after retroviral infection (25). COX-2 expression and PGE_2 production are also known to be involved in the inhibitory mechanism of Treg cells (26). To further elucidate the role of COX enzymes in Th17 cell differentiation, the expression of COX-1 and COX-2 in $CD4^+$ T cells during Th17 cell differentiation *in vitro* was investigated. Naive $CD4^+$ T cells purified from WT mouse spleens were stimulated for 4–5 days to induce Th17 cell differentiation, and COX-1 and COX-2 mRNA and protein levels were examined by real-time polymerase chain reaction (RT-PCR) and immunoblotting, respectively. Untreated, naive $CD4^+$ T cells expressed low levels of COX-2 mRNA and protein (Figures 3A–3D). Treatment of naive $CD4^+$ T cells with anti-CD3, anti-CD28, anti–IFN-γ, TGF-β, and IL-6 increased COX-2 mRNA and protein levels in an IL-6 dose-dependent fashion. In contrast, COX-1 was constitutively expressed in naive $CD4^+$ T cells and mRNA and protein levels were unchanged during Th17 cell differentiation (Figures 3A–3D). We also used flow cytometry to measure intracellular expression of COX-1 and COX-2 during Th17 differentiation. IL-6 significantly and dose-dependently increased the expression of COX-2 protein, but not COX-1 protein, in Th17 cells differentiated from naive $CD4^+$ T cells isolated from spleen (Figures 3E and 3F) and lung (Figures 3G and 3H). To provide further evidence of COX-2 expression in $CD4^+$ T cells that is linked to regulation of Th17 cells *in vivo*, we isolated $CD4^+$ T cells from lung after ovalbumin exposure and examined COX-2 expression by RT-PCR. The results showed that COX-2 mRNA levels were significantly increased in lung $CD4^+$ cells after ovalbumin exposure (Figure 3I). Together, these results demonstrate that COX-2 is up-regulated during Th17 cell differentiation *in vitro* and *in vivo*.

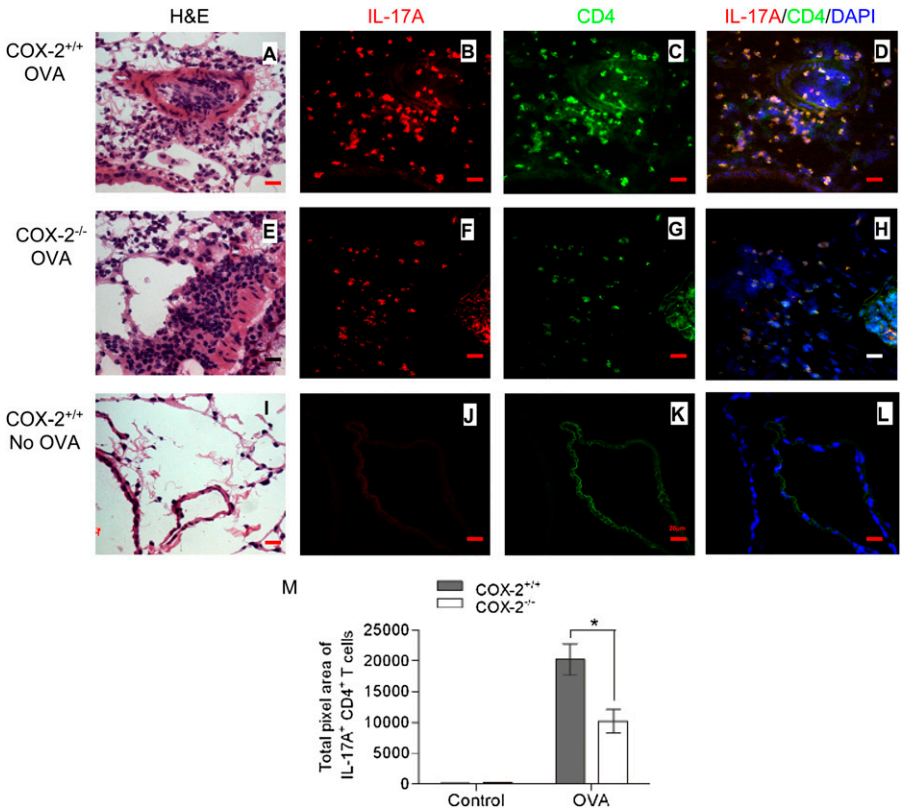

Figure 2. Reduced Th17 cells in lung of cyclooxygenase (COX)-$2^{-/-}$ mice after ovalbumin (OVA) exposure. COX-$2^{+/+}$ and COX-$2^{-/-}$ mice were sensitized with OVA in adjuvant (or given adjuvant alone). Fourteen to 21 days later, mice were exposed to inhaled OVA (or inhaled phosphate-buffered saline) for 4 consecutive days. Forty-eight hours after final OVA (or phosphate-buffered saline) exposure, lung tissue sections were stained with hematoxylin and eosin (H&E) and visualized by light microscopy (*A*, *E*, and *I*) (original magnification ×100). Visualization of Th17 cells in COX-$2^{+/+}$ and COX-$2^{-/-}$ lung tissue sections was accomplished by immunofluorescence staining with phycoerythrin (PE)-labeled anti–IL-17A (*B*, *F*, and *J*) and fluorescein isothiocyanate (FITC)–labeled anti-CD4 (*C*, *G*, and *K*) antibodies. *D*, *H*, and *L* are merged images of anti–IL-17A and PE, anti–CD4 and FITC, and DAPI. All immunofluorescent images are shown at original magnification ×60; numerical aperture, 1.4; scale bar, 25 μm. Results are representative of three independent experiments. Multiple (n = 22–29) randomly selected regions (1,300 × 1,030 $pixel^2$ = 350 × 277 μm^2) from each lung section were counted and quantified by a masked observer using MetaMorph software (*M*); *$P < 0.05$ versus COX-$2^{+/+}$.

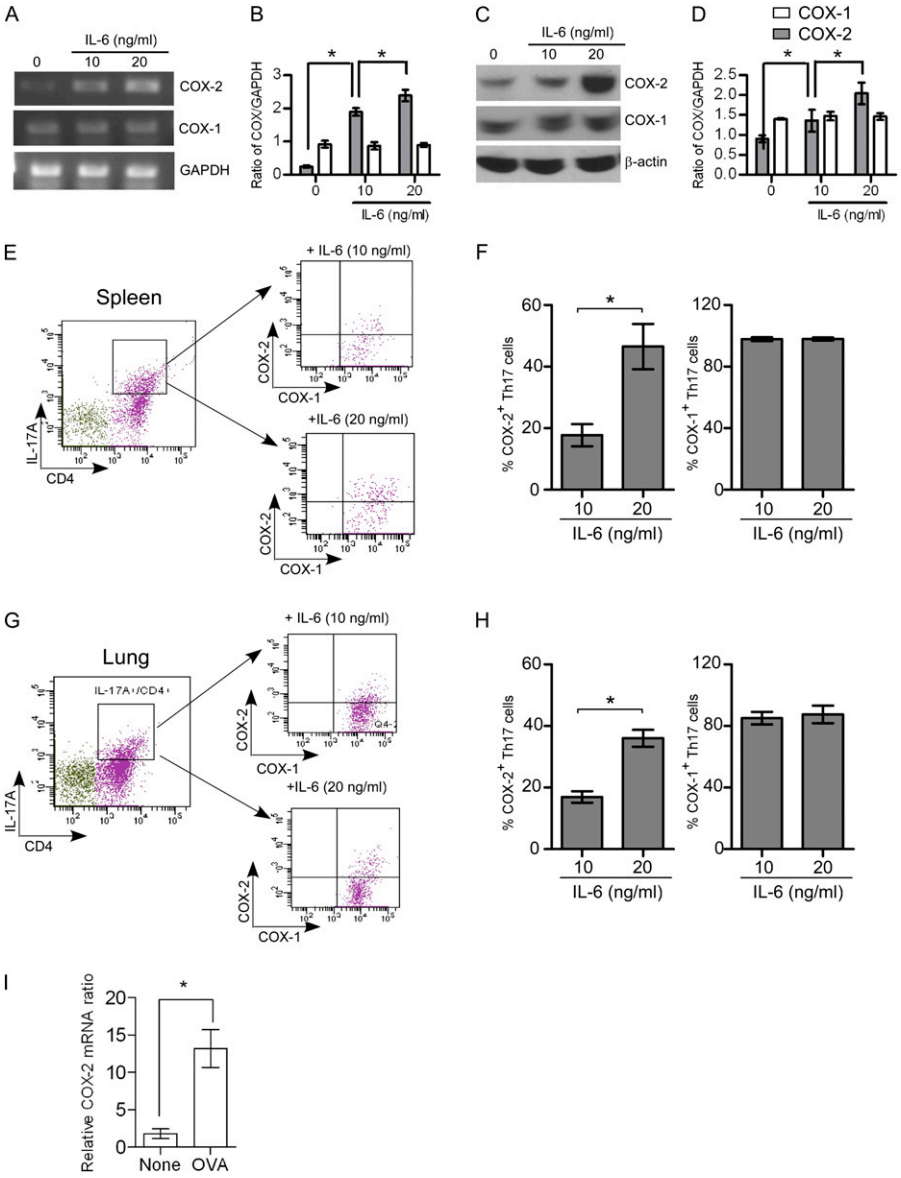

Figure 3. Regulation of cyclooxygenase (COX)-1 and COX-2 during Th17 cell differentiation from naive CD4$^+$ T cells. Naive CD4$^+$ T cells isolated from spleen were stimulated with or without anti-CD3 (3 μg/ml), anti-CD28 (3 μg/ml), anti–INF-γ (3 μg/ml), transforming growth factor-β (10 ng/ml), and IL-6 (10 or 20 ng/ml) for 4 days to induced Th17 differentiation and then the cells were analyzed for COX-1 and COX-2 expression by real-time polymerase chain reaction (RT-PCR), protein immunoblotting, and flow cytometry. (*A*) PCR products showing mRNA levels of COX-1, COX-2, and glyceralhehyde phosphate dehydrogenase (GAPDH). (*B*) COX-1 and COX-2 mRNAs were quantified by densitometry and expressed as the density ratio of COX-1 or COX-2 relative to GAPDH. Results are representative of three independent experiments. (*C*) Immunoblot analysis of cell lysates for COX-1, COX-2, and β-actin. (*D*) Protein levels of COX-1 and COX-2 were quantified by densitometry and expressed relative to β-actin. Results are representative of three independent experiments. (*E* and *G*) Naive CD4$^+$ T cells were stimulated with anti-CD3, anti-CD28, anti–INF-γ, transforming growth factor-β, and IL-6 (10 and 20 ng/ml) for 4 days, and then stimulated for 4 hours with 12–0-tetradecanoyl-phorbol-13-acetate and ionomycin (500 ng/ml each) in the presence of brefeldin A (1 μg/ml) before intracellular staining with anti-CD4, IL-17, COX-1, and COX-2. Gating on the CD4$^+$ IL-17A$^+$ cell population, COX-1 and COX-2 expression was analyzed by flow cytometry. (*F* and *H*) The percent of the COX-1– and COX-2–positive Th17 cells after treatment with different concentrations of IL-6 is shown. Results are representative of three independent experiments. *$P < 0.05$. (*I*) Lung CD4$^+$ T cells were isolated after ovalbumin (OVA) exposure *in vivo* and COX-2 mRNA levels were quantified by RT-PCR; n = 6; *$P < 0.01$ versus control (no OVA).

Impaired Th17 Cell Differentiation of COX-2$^{-/-}$ Naive CD4$^+$ T Cells *In Vitro*

To examine the role of COX-2 in Th17 cell differentiation *in vitro*, we treated equal numbers of naive CD4$^+$ T cells (>95% pure) isolated from WT and COX-2$^{-/-}$ mouse spleens with anti-CD3, anti-CD28, anti–IFN-γ, TGF-β, and IL-6, and quantified IL-17–producing CD4$^+$ T cells by flow cytometry on Day 4. Interestingly, we observed that 6.6% of WT naive CD4$^+$ T cells differentiated into Th17 cells, compared with only 0.7% of COX-2$^{-/-}$ naive CD4$^+$ T cells (Figures 4A and 4B). Thus, there was significantly impaired Th17 cell differentiation of COX-2$^{-/-}$ CD4$^+$ T cells *in vitro*. Consistent with the role of COX-2 in Th17 cell differentiation, the selective COX-2 inhibitor NS-398 also significantly reduced Th17 differentiation *in vitro* (Figure 4C).

We also examined the kinetics of Th17 cell differentiation of WT and COX-2$^{-/-}$ naive CD4$^+$ T cells. Th17 cell differentiation of WT naive CD4$^+$ T cells increased daily and attained a maximum on Day 4 of treatment (Figure 4D). In contrast, there was no significant peak of Th17 cell differentiation in COX-2$^{-/-}$ naive CD4$^+$ T cells. Consistent with these data, IL-17A production also peaked on Day 4 in WT naive CD4$^+$ T cells (Figure 4E). In contrast, IL-17A production remained low in COX-2$^{-/-}$ naive CD4$^+$ T cells treated under identical conditions.

IL-6 critically regulates the differentiation potential of naive T cells into Th17 cells. Moreover, allergic COX-2$^{-/-}$ mice have reduced levels of IL-6 (Figures 1I and 1J). Therefore, we tested whether alterations in IL-6 levels were responsible for defective Th17 cell differentiation of COX-2$^{-/-}$ naive CD4$^+$ T cells. We found that Th17 cell differentiation of COX-2$^{+/+}$ naive CD4$^+$ T cells (Figure 4F) and the ability of these cells to produce IL-17A (Figure 4G) were IL-6 dosage-dependent; however, IL-6 treatment failed to increase Th17 cell differentiation or IL-17A production of COX-2$^{-/-}$ naive CD4$^+$ T cells to levels observed in COX-2$^{+/+}$ cells (Figures 4F and 4G). Thus, the reduced Th17 cell differentiation in COX-2$^{-/-}$ naive CD4$^+$ T cells does not seem to be caused by reduced IL-6 production.

To exclude the possibility that the Th17 differentiation defect in COX-2$^{-/-}$ mice was caused by altered IL-6 or TGF-β receptor expression in naive CD4$^+$ T cells, we examined the expression of IL-6 and TGF-β receptors by RT-PCR in COX-2$^{-/-}$ naive CD4$^+$ T cells and in Th17 cells *in vitro*. The results show similar expression of IL-6 and TGF-β receptors in both naive

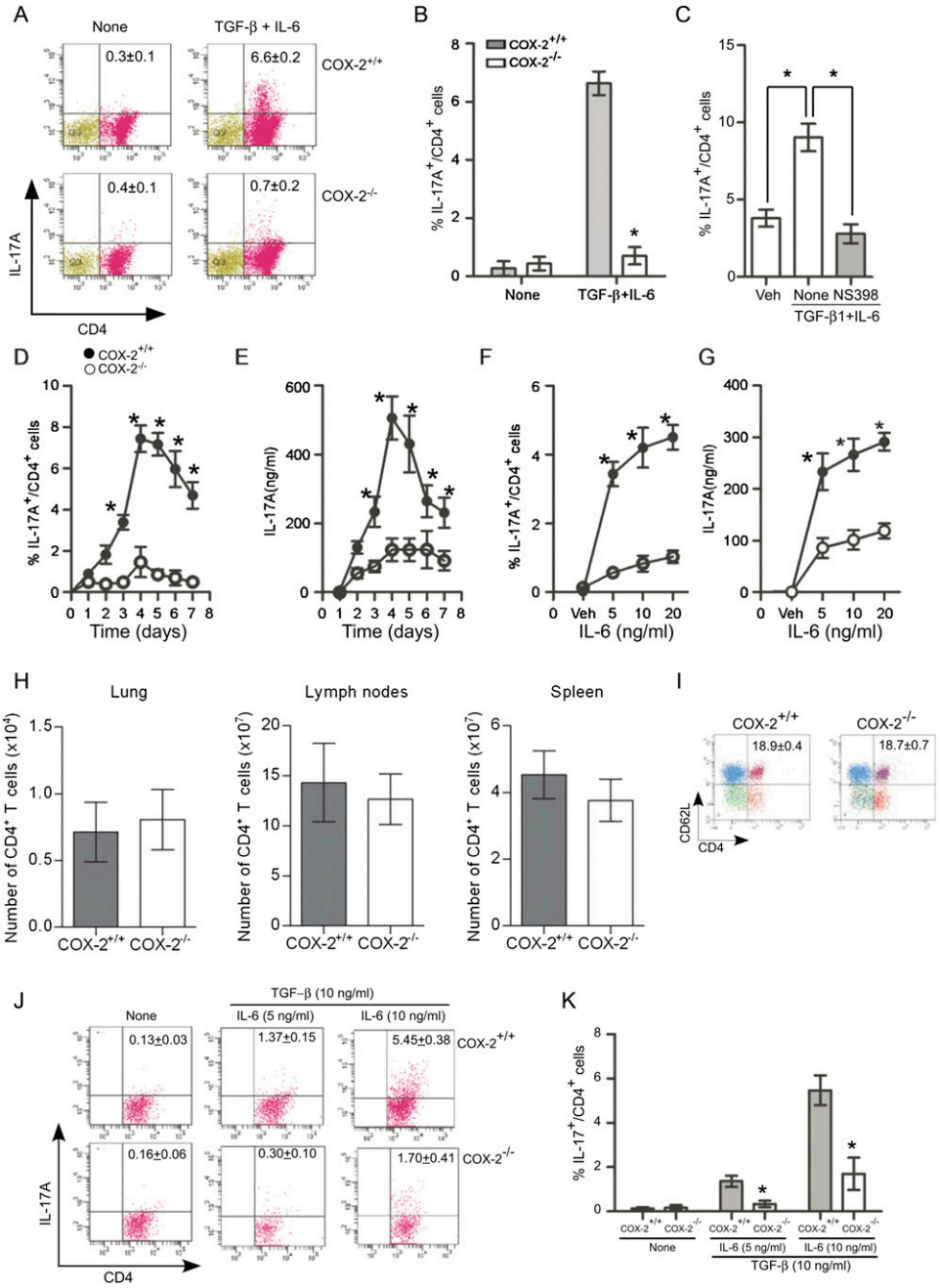

Figure 4. Impaired Th17 differentiation in cyclooxygenase (COX)-2$^{-/-}$ naive CD4^{+} T cells *in vitro*. (*A*) Naive CD4^{+} T cells isolated from spleens of COX-2$^{+/+}$ and COX-2$^{-/-}$ mice were treated with or without anti-CD3 (3 μg/ml), anti-CD28 (3 μg/ml), anti–INF-γ (3 μg/ml), transforming growth factor (TGF)-β (10 ng/ml), and IL-6 (10 ng/ml) for 4 days. The cells were then stimulated for 4 hours with 12–0-tetradecanoyl-phorbol-13-acetate and inomycin (500 ng/ml each) in the presence of brefeldin A (1 μg/ml) before flow cytometry analysis. Data are representative of three independent experiments. (*B*) The percentage of naive CD4^{+} T cells from COX-2$^{+/+}$ and COX-2$^{-/-}$ mice that differentiated to CD4^{+} IL-17A^{+} (Th17) cells is shown. (*C*) Inhibition of COX-2 with NS-398 during Th17 cell differentiation from naive CD4^{+} T cells was examined. Naive CD4^{+} T cells were cultured with anti-CD3 (3 μg/ml), anti-CD28 (3 μg/ml), anti–INF-γ (3 μg/ml), TGF-β (10 ng/ml), and IL-6 (10 ng/ml) in the presence or absence of NS-398 (20 μM) for 4 days. The cells were then stimulated for 4 hours with 12–0-tetradecanoyl-phorbol-13-acetate and inomycin (500 ng/ml each) in the presence of brefeldin A (1 μg/ml) before flow cytometry analysis. (*D*) The percentage of IL-17A^{+} CD4^{+} cells was examined by flow cytometry at different time points (Days 1–7) after treatment with anti-CD3, anti-CD28, anti–INF-γ, TGF-β, and IL-6. (*E*) Cell culture supernatants from *D* were collected at different time points and IL-17A levels were assayed by ELISA. (*F*) Naive CD4^{+} T cells from COX-2$^{+/+}$ and COX-2$^{-/-}$ mice were differentiated to Th17 cells in the presence of different amounts of IL-6 (0, 5, 10, and 20 ng/ml) for 4 days and the percent of CD4^{+} IL-17A^{+} cells was analyzed by flow cytometry. (*G*) IL-17A levels in cell culture supernatants from *F* were assayed by ELISA. For panels *C–F*, data are representative of three independent experiments; closed circles = COX-2$^{+/+}$; open circles = COX-2$^{-/-}$; * $P < 0.05$ versus COX-2$^{+/+}$. (*H*) Total number of CD4^{+} T cells were enumerated in spleen, lymph nodes, and lung from COX-2$^{+/+}$ and COX-2$^{-/-}$ mice (n = 10 per group). (*I*) Splenocytes from COX-2$^{+/+}$ and COX-2$^{-/-}$ mice were analyzed for CD62L^{+} and CD4^{+} expression. Representative scattergrams are illustrated and the percentages of CD62L^{+} CD4^{+} cells (mean ± SE; n = 10 per group) are shown. (*J*) Naive CD4^{+} CD62L^{+} T cells from spleens of COX-2$^{+/+}$ and COX-2$^{-/-}$ mice were first sorted by flow cytometry. These highly purified naive CD4^{+} CD62L^{+} T cells were then differentiated into Th17 cells as described in *A*. On Day 4, the cells were collected and the percentage of IL-17A^{+} CD4^{+} cells was analyzed by flow cytometry. (*K*) Quantification of Th17 cell differentiation of COX-2$^{+/+}$ and COX-2$^{-/-}$ naive CD4^{+} CD62L^{+} T cells is shown.

CD4^{+} T cells and Th17 cells from COX-2$^{-/-}$ and WT mice (Figure E3).

To exclude the possibility that the defect of Th17 cell differentiation in COX-2$^{-/-}$ mice was caused by diminished numbers of naive CD4^{+} T cells, we examined CD4^{+} T cell populations in various tissues from COX-2$^{+/+}$ and COX-2$^{-/-}$ mice by flow cytometry. The number of CD4^{+} T cells in spleens, lymph nodes, and lungs were comparable between COX-2$^{-/-}$ and COX-2$^{+/+}$ mice (Figure 4H). Furthermore, the percentage of splenic CD62L^{+} CD4^{+} T cells was similar between COX-2$^{-/-}$ and COX-2$^{+/+}$ mice (Figure 4I).

B cells can produce a variety of cytokines that influence Th17 differentiation (27). To exclude the possibility that the defect of Th17 cell differentiation in COX-2$^{-/-}$ mice was caused by impaired B-cell development, we examined B220^{+} populations in various tissues from COX-2$^{+/+}$ and COX-2$^{-/-}$ mice. The number of B220^{+} cells in blood, spleens, lymph nodes, BALF, and lungs were also comparable between COX-2$^{-/-}$ and COX-2$^{+/+}$ mice (Figure E4).

Defective Th17 cell differentiation in COX-2$^{-/-}$ mice could also be caused by alterations in other cell types, such as dendritic cells. These cells produce a variety of cytokines that can activate

Stat3 and initiate RORγt transcription and Th17 cell differentiation (28). Moreover, PGs secreted from dendritic cells might stimulate Th17 cell differentiation via a paracrine mechanism. Alternatively, PGs secreted by T cells might stimulate dendritic cells to produce IL-23, which can promote differentiation of Th17 cells (20, 29). To eliminate the possibility that contamination of naive $CD4^+$ T cells by other cell types contributed to the Th17 cell differentiation defect in COX-$2^{-/-}$ mice, we sorted naive $CD4^+$ $CD62L^+$ T cells from spleens of COX-$2^{+/+}$ and COX-$2^{-/-}$ mice by flow cytometry. These highly (>99%) purified cells were then differentiated into Th17 cells *in vitro*. Consistent with the results described in Figure 4, the percentage of highly purified naive $CD4^+$ $CD62L^+$ T cells from COX-$2^{-/-}$ mice that differentiated into Th17 cells was significantly lower than from COX-$2^{+/+}$ mice (Figures 4J and 4K). These data suggest that COX-2 products act as T-cell autocrine factors to induce Th17 differentiation.

COX-$2^{-/-}$ Th17 Cells Have Altered Stat3 Phosphorylation and RORγt Expression

Th17 cell differentiation is known to be accompanied by Stat3 activation and induction of the lineage-specific transcription factor RORγt (30). To determine if the defect in Th17 cell differentiation in COX-$2^{-/-}$ mice was accompanied by altered Stat3 activation and RORγt expression, we examined Stat3 phosphorylation and RORγt mRNA levels during Th17 differentiation *in vitro*. Stat3 phosphorylation was significantly reduced in Th17 cells differentiated from $CD4^+$ T cells isolated from COX-$2^{-/-}$ mice relative to those isolated from COX-$2^{+/+}$ mice (Figures 5A and 5B). Likewise, the expression of RORγt was markedly lower in Th17 cells differentiated from naive $CD4^+$ T cells isolated from COX-$2^{-/-}$ spleens compared with those isolated from COX-$2^{+/+}$ spleens (Figures 5C and 5D).

COX-2 Regulates Th17 Cell Differentiation through PGs

To further examine the role of COX-2–derived PGs in regulating T-cell differentiation, eicosanoid levels in supernatants of naive $CD4^+$ T cells and *in vitro* differentiated Th17 cells from COX-$2^{+/+}$ and COX-$2^{-/-}$ mice were measured by liquid chromatography–tandem mass spectroscopy. PG levels were low-undetectable in naive $CD4^+$ T cells and not significantly different between the two genotypes (Figure 5E). Levels of each of the PGs increased significantly with Th17 differentiation in cells from COX-$2^{+/+}$ mice; the largest increases were in PGE_2, $PGF_{2\alpha}$, and 6-keto-$PGF_{1\alpha}$ (the stable prostacyclin metabolite). Importantly, levels of each of the PGs were reduced in Th17 cells differentiated from $CD4^+$ T cells isolated from COX-$2^{-/-}$ mice relative to COX-$2^{+/+}$ mice (Figure 5E). In addition, immunofluorescent staining showed that Th17 cells from COX-$2^{+/+}$ mouse lungs produced $PGF_{2\alpha}$ and PGI_2 after ovalbumin sensitization and exposure *in vivo* (Figure E5). Together, these data suggest that PG production is increased during Th17 differentiation and that COX-2 is critical for optimal PG production in Th17 cells.

To determine if the decreased PG production in COX-$2^{-/-}$ Th17 cells resulted in altered systemic levels of these eicosanoids or altered levels in the allergic lung, we measured PG levels in blood and BAL fluid of WT and COX-$2^{-/-}$ mice after ovalbumin sensitization and exposure *in vivo*. Consistent with our prior published data (16, 23), we observed that PG levels in blood and BAL fluid were not significantly altered in allergic COX-$2^{-/-}$ mice relative to WT (Figure E6). Thus, although COX-2 is critical for optimal production of PGs in Th17 cells, it does not seem to contribute significantly to circulating levels of PGs or to PG production at the whole organ level.

Next, we determined whether PGs could restore the Th17 cell differentiation and IL-17A production defects in COX-$2^{-/-}$ mice. Synthetic PGs (1 μM each) were added to naive $CD4^+$ T cells during Th17 cell differentiation *in vitro* and the percent of Th17 cells and IL-17A production were measured by flow cytometry and ELISA, respectively. Administration of synthetic $PGF_{2\alpha}$ and PGI_2, but not PGD_2 or PGE_2, partially restored Th17 cell differentiation (Figure 5F) and IL-17A production (Figure 5G) in COX-$2^{-/-}$ cells. $PGF_{2\alpha}$ and PGI_2 also increased Th17 differentiation in COX-$2^{+/+}$ cells, although these changes did not reach statistical significance. In contrast, PGD_2 decreased Th17 differentiation and IL-17A production in COX-$2^{+/+}$ cells (Figures 5F and 5G). To examine the specificity of cellular responses to the prostanoids, we performed a dose-response study of $PGF_{2\alpha}$, PGI_2, and PGE_2 on Th17 cell differentiation *in vitro* over a range of prostanoid concentrations (50 nM to 1 μM). Both PGI_2 and $PGF2_\alpha$ significantly enhanced Th17 cell differentiation at concentrations as low as 500 nM and showed a trend for enhanced Th17 cell differentiation at lower concentrations (Figure 5H). In contrast, we did not observe a significant effect of PGE_2 on Th17 cell differentiation at concentrations as high as 1 μM. Importantly, synthetic $PGF_{2\alpha}$ and PGI_2 treatment increased Stat3 phosphorylation in naive $CD4^+$ T cells from WT mice *in vitro*, whereas Stat3 activation by PGD_2 and PGE_2 was not observed (Figures 5I and 5J; Figure E7). Likewise, $PGF_{2\alpha}$ and PGI_2, but not PGD_2 or PGE_2, significantly induced RORγt expression in *in vitro* differentiated COX-$2^{-/-}$ Th17 cells (Figures 5K and 5L). These results suggest that COX-2 directly regulates Th17 cell differentiation *in vitro* through production of specific PGs.

We then implanted osmotic minipumps containing synthetic PGs to determine if we could restore the COX-$2^{-/-}$ Th17 cell defect *in vivo* in allergic mice after ovalbumin sensitization and exposure. Th17 cell percentages were increased in lungs, BALF, and lymph nodes of allergic COX-$2^{-/-}$ mice treated with iloprost (a stable PGI_2 analog), $PGF_{2\alpha}$, PGE_2, or a combination of iloprost, $PGF_{2\alpha}$, and PGE_2 compared with mice treated with vehicle (Figures 6A–6C). Consistent with these data, IL-17A levels in BALF were significantly increased in COX-$2^{-/-}$ mice treated with iloprost, $PGF_{2\alpha}$, PGE_2, or the combination (Figure 6D). Taken together, these data suggest that COX-2–derived PGs regulate Th17 cell differentiation and IL-17A production *in vivo* in allergic lung disease.

PGs Regulate Th17 Cell Differentiation through Their Cognate Receptors

To determine whether COX-2–derived PGs regulate Th17 cell differentiation through their cognate receptors, we first determined the expression of the prostanoid receptors on naive $CD4^+$ T cells isolated from allergic COX-$2^{+/+}$ and COX-$2^{-/-}$ mice after *in vivo* ovalbumin sensitization and exposure. The EP1, EP4, DP1, DP2, and FP receptors were present on a low percentage of naive $CD4^+$ T cells isolated from spleen, lymph nodes, lung, blood, and BALF of allergic COX-$2^{+/+}$ and COX-$2^{-/-}$ mice; there were no significant differences in the percentage of naive $CD4^+$ T cells that expressed these receptors between the two genotypes (Figure 7A). The EP2, EP3, and IP receptors were present on a significantly greater percentage of naive $CD4^+$ T cells isolated from allergic COX-$2^{+/+}$ and COX-$2^{-/-}$ mouse tissues, but again there were no consistent differences between the two genotypes (Figure 7A).

We next examined PG receptor expression at the mRNA level by RT-PCR on both naive $CD4^+$ T cells and on *in vitro* differentiated Th17 cells from COX-$2^{+/+}$ and COX-$2^{-/-}$ mice. Naive $CD4^+$ splenic T cells expressed EP1, EP2, EP3, and EP4 receptor mRNAs, and to a lesser extent DP, FP, and IP receptor

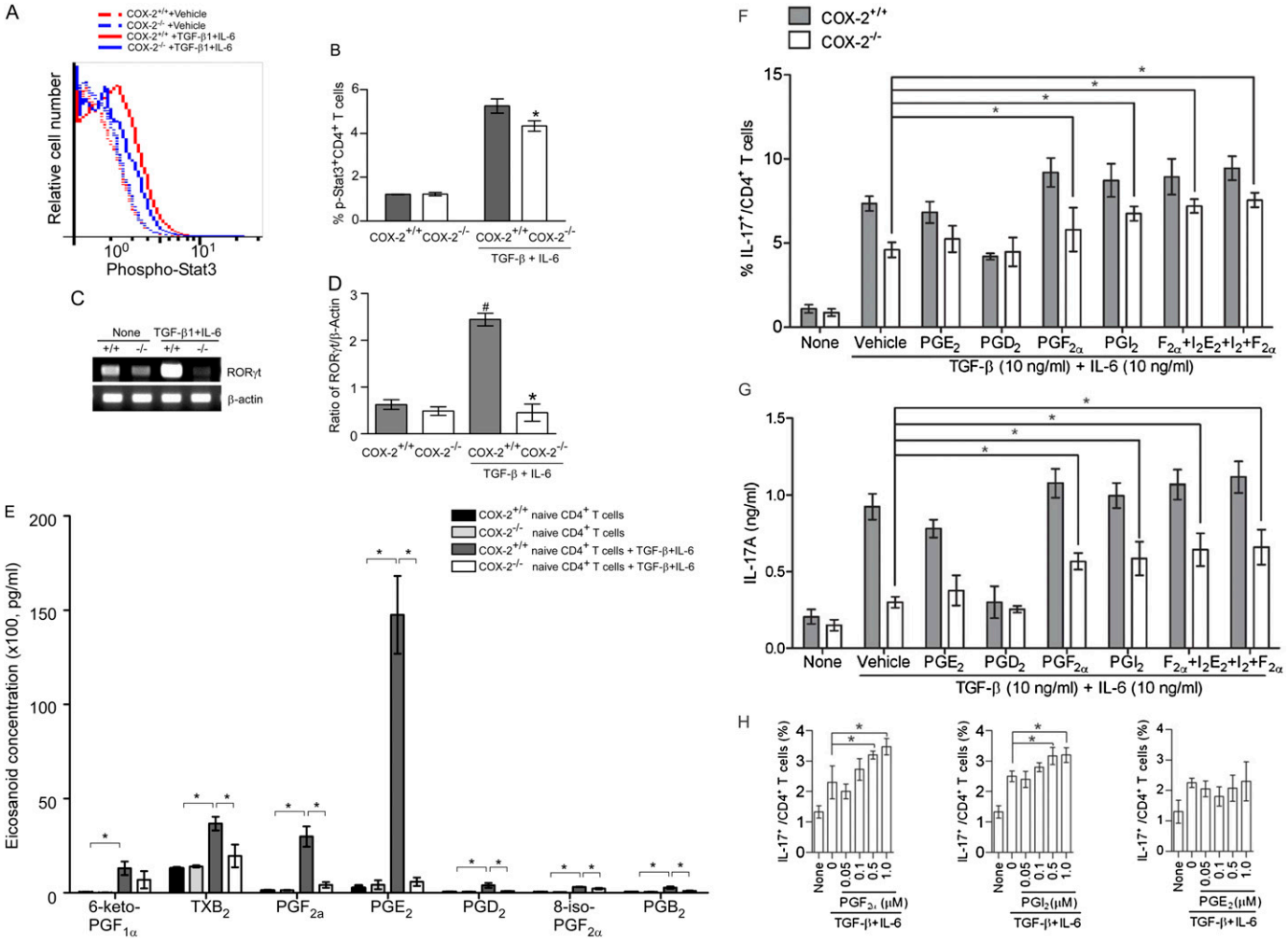

Figure 5. Cyclooxygenase (COX)-2–derived prostaglandins (PGs) regulate Th17 cell differentiation *in vitro*. (*A*) Stat3 phosphorylation during Th17 differentiation of $CD4^+$ T cells from COX-$2^{+/+}$ and COX-$2^{-/-}$ mice was assayed using anti–phospho-stat3 (pY705)-PE by flow cytometry. (*B*) Ratio of phospho-Stat3/total Stat3 during Th17 cell differentiation of COX-$2^{+/+}$ and COX-$2^{-/-}$ naive $CD4^+$ T cells. (*C*) RORγt mRNA expression in differentiated $CD4^+$ T cells from COX-$2^{+/+}$ and COX-$2^{-/-}$ mice was analyzed by real-time polymerase chain reaction. (*D*) Ratio of RORγt to β-actin mRNA during Th17 cell differentiation of COX-$2^{+/+}$ and COX-$2^{-/-}$ naive $CD4^+$ $CD62L^+$T cells. All data are representative of three independent experiments; $^{\#}P < 0.05$ versus COX-$2^{+/+}$ naive $CD4^+$ T cells; $^*P < 0.05$ versus COX-$2^{+/+}$. (*E*) Eicosanoid levels in supernatants of naive $CD4^+$ T cells and *in vitro* differentiated Th17 cells from COX-$2^{+/+}$ and COX-$2^{-/-}$ mice were measured by liquid chromatography/tandem mass spectroscopy. Results are representative of five independent experiments. Effect of synthetic PGs on Th17 cell differentiation of (*F*) and IL-17A production by (*G*) naive $CD4^+$ T cells isolated from COX-$2^{+/+}$ and COX-$2^{-/-}$ mice *in vitro*. PGs were added at 1 μM on Days 2 and 3 of culture, and culture supernatants were collected on Day 4. IL-17A was measured by ELISA. Results are representative of three independent experiments; $^*P < 0.05$ versus vehicle. (*H*) Dose dependency of PGI_2, $PGF_{2\alpha}$, and PGE_2 (50 nM to 1 μM) on Th17 cell differentiation was examined *in vitro*; n = 4; $^*P < 0.05$ versus no PG. (*I* and *J*) Phospho-Stat3 and total Stat3 levels were determined by immunoblotting before and 5–60 minutes after treatment of naive $CD4^+$ T cells with synthetic PGs (1 μM each). Results are representative of three independent experiments; $^*P < 0.05$ versus time 0. (*K* and *L*) RORγt mRNA levels measured by real-time polymerase chain reaction in *in vitro* differentiated Th17 cells treated with or without synthetic PGs (1 μM each). Results are representative of three independent experiments; $^*P < 0.05$ versus vehicle.

mRNAs (Figure 7B). There were no substantive differences in mRNA levels between COX-$2^{+/+}$ and COX-$2^{-/-}$ mice. All of the PG receptor mRNAs were detected in *in vitro* differentiated Th17 cells (Figure 7B). Levels of EP1, EP2, and DP receptor mRNAs were lower in COX-$2^{-/-}$ Th17 cells compared with COX-$2^{+/+}$ Th17 cells. Thus, the FP and IP receptors were induced in both COX-$2^{+/+}$ and COX-$2^{-/-}$ cells during Th17 cell differentiation, whereas the DP receptor was not induced in COX-$2^{-/-}$ cells.

To elucidate further the role of PG receptors in Th17 cell differentiation, we examined the effects of selective FP and IP receptor antagonists and siRNA knockdown *in vitro*. Th17 cell differentiation of naive $CD4^+$ T cells was significantly reduced if either the selective FP receptor antagonist AL8810 or the selective IP receptor antagonist CAY10441 was added to the culture medium (Figure 7C). Likewise, siRNA knockdown of either the FP receptor or the IP receptor significantly reduced Th17 cell differentiation *in vitro* (Figure 7D; Figure E8). These results indicate that COX-2–derived PGs directly regulate Th17 cell differentiation through both the FP and IP receptors.

DISCUSSION

Th17 cells have been recognized as a unique subset of effector T cells that are distinct from Th1 and Th2 subsets (31–33). They

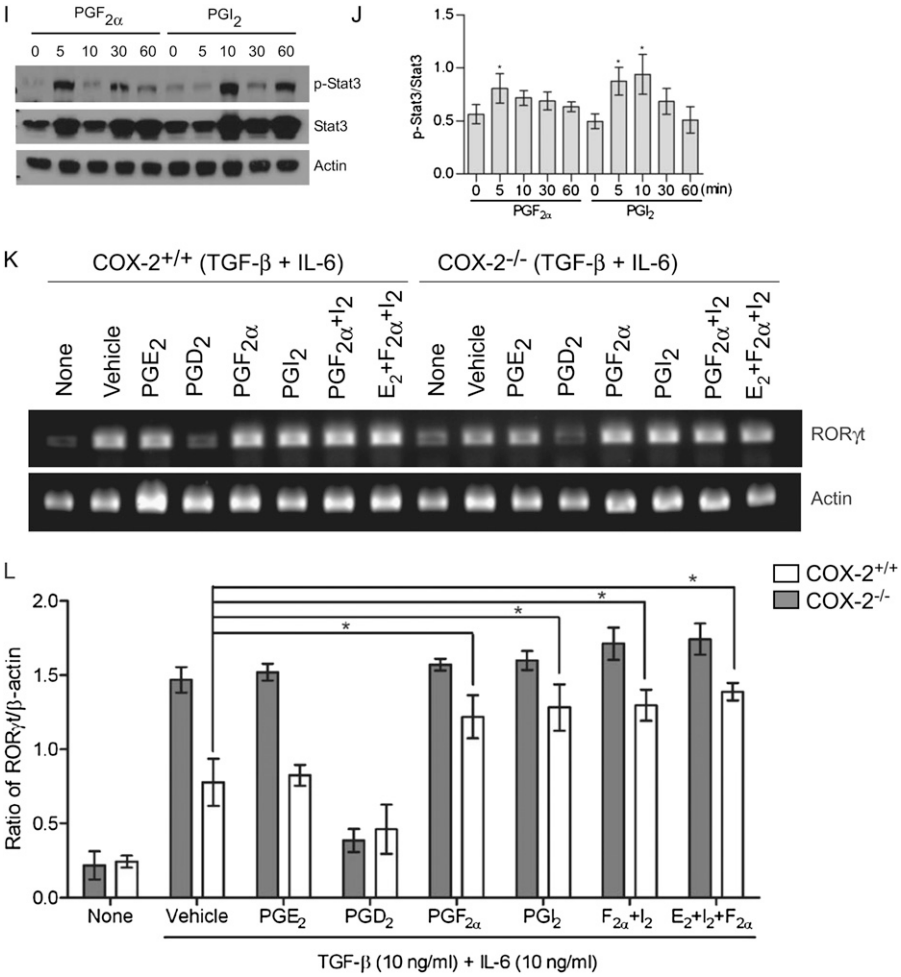

Figure 5. (*Continued*).

have been implicated as potent effectors of autoimmune disorders, such as multiple sclerosis, psoriasis, arthritis, and inflammatory bowel disease, and allergic disorders, such as asthma (2, 34, 35). COX-derived PGs are known to play important roles in the regulation of inflammatory responses. However, the role of PGs in regulating Th17 cell differentiation and function has remained enigmatic. In this report, we investigated the functions of COX-1 and COX-2 in regulating Th17 cell differentiation and function in an allergic lung inflammation model. Our main findings are as follows: (*1*) the number of Th17 cells in lung, BALF, and lymph nodes, and the levels of IL-17A in blood and BALF, are decreased in allergic COX-$2^{-/-}$ mice *in vivo*; (*2*) COX-2 expression is induced during Th17 cell differentiation in an IL-6 dose-dependent manner; (*3*) Th17 cell differentiation of naive $CD4^+$ T cells from COX-$2^{-/-}$ mice is impaired *in vitro*; (*4*) selective COX-2 inhibition also inhibits Th17 cell differentiation; (*5*) activation of Stat3 and induction of RORγt are decreased during Th17 cell differentiation in COX-$2^{-/-}$ mice; (*6*) the production of PGs is increased during Th17 differentiation; (*7*) PG production during Th17 cell differentiation is lower in COX-$2^{-/-}$ mice; (*8*) synthetic PGs partially restore the Th17 cell differentiation defect in COX-$2^{-/-}$ mice *in vitro* and *in vivo*; and (*9*) PGs act as autacoids to promote Th17 cell differentiation by acting through their cognate receptors. Together, these data indicate that COX-2, but not COX-1, is a critical regulator of local cytokine production and Th17 cell differentiation during allergic lung inflammation.

Immune cells produce a variety of PGs that have both proinflammatory and antiinflammatory effects (36). PGE_2 is the most abundantly produced prostanoid in the body and has been shown to play an important role in regulating inflammatory

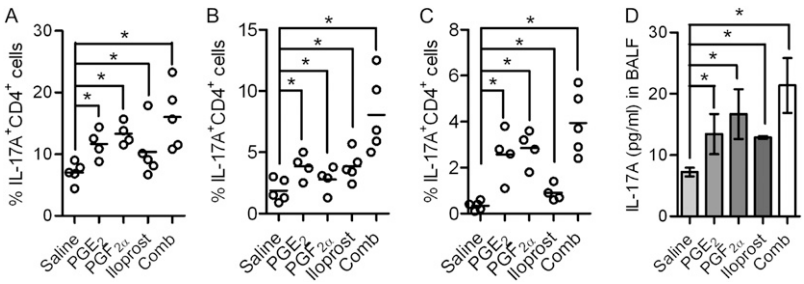

Figure 6. Administration of synthetic prostaglandins (PGs) restores Th17 cell percentages in allergic cyclooxygenase (COX)-$2^{-/-}$ mice *in vivo*. Alzet minipumps filled with PGE_2, $PGF_{2\alpha}$, iloprost, or the combination of PGs were implanted into sensitized COX-$2^{-/-}$ mice. After 1 week, mice were exposed to ovalbumin daily for 4 days. Forty-eight hours after the last ovalbumin exposure, the mice were killed and the percentages of IL-17A^+ $CD4^+$ T cells in lung (*A*), bronchoalveolar lavage fluid (BALF) (*B*), and lymph nodes (*C*) were determined by flow cytometry. (*D*) IL-17 levels in BALF samples from the above mice were assayed for IL-17A by ELISA. Results are representative of three independent experiments. n = 4; $^*P < 0.05$ versus saline vehicle. In *A*, *B*, and *C*, lines indicate the mean, and each symbol represents an individual mouse.

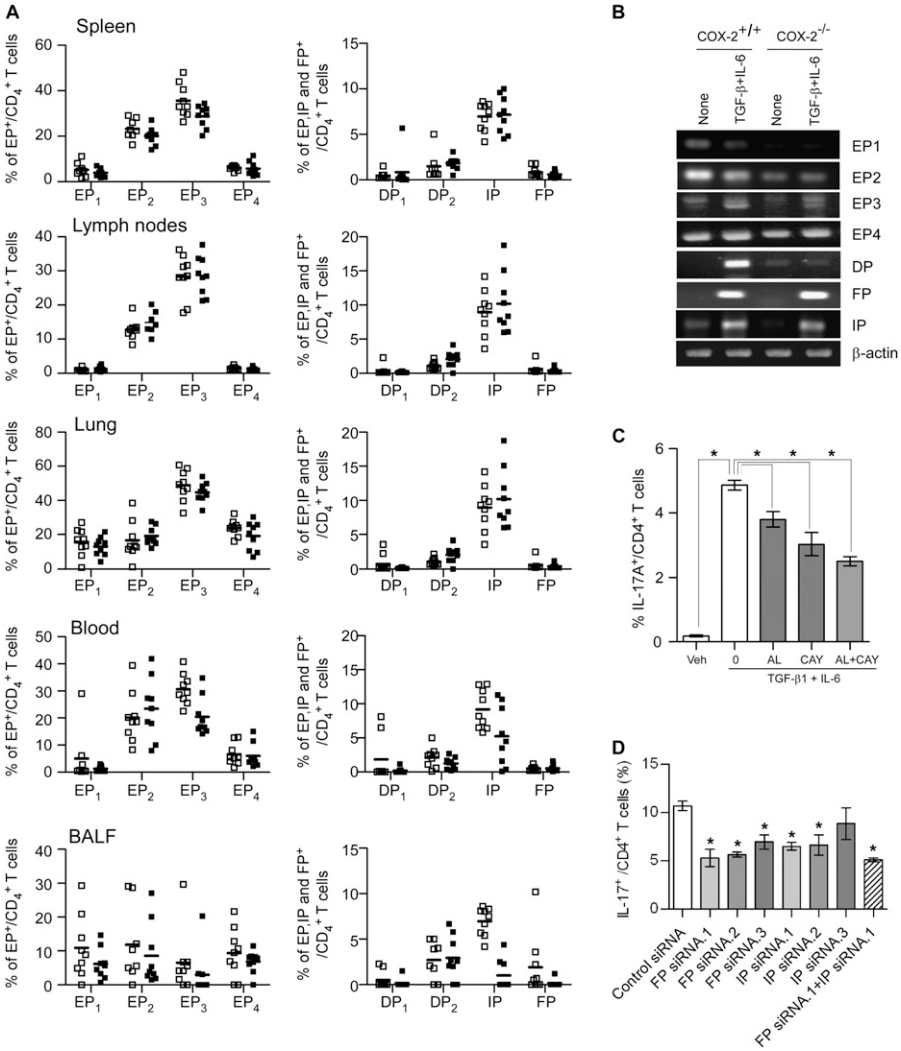

Figure 7. Expression of prostaglandin (PG) receptors on CD4$^+$ T cells *in vivo* and *in vitro* differentiated Th17 cells from cyclooxygenase (COX)-2$^{+/+}$ and COX-2$^{-/-}$ mice. (*A*) The percentage of CD4$^+$ T cells isolated from spleen, lymph nodes, lung, blood, and bronchoalveolar lavage fluid (BALF) of allergic COX-2$^{+/+}$ and COX-2$^{-/-}$ mice was determined by flow cytometry. Antibodies to each of the PG receptors were conjugated with AlexaFlur-488 or Alexa-Flur-594 using a Therm Scientific DyLightTM 488/594 microscale antibody labeling kit. Cell suspensions were prepared and stained with the conjugated receptor antibodies. EP1-EP4, DP$_1$, DP$_2$, FP, and IP receptor-positive CD4$^+$ T cells were quantified by flow cytometry by gating on the IL-17$^+$ CD4$^+$ T cell population. Lines indicate the mean, and each symbol (*open squares*, COX-2$^{+/+}$; *solid squares*, COX-2$^{-/-}$) represents an individual mouse. (*B*) Levels of EP1-EP4, DP, FP, and IP receptor mRNAs in naive CD4$^+$ T cells and in *in vitro* differentiated Th17 cells from COX-2$^{+/+}$ and COX-2$^{-/-}$ mice. Naive CD4$^+$ T cells isolated from COX-2$^{+/+}$ and COX-2$^{-/-}$ mice were stimulated with or without anti-CD3, anti-CD28, anti–INF-γ, IL-6, and transforming growth factor (TGF)-β for 4 days. Total RNA was then extracted and reverse transcribed to cDNA for detection of EP1-EP4, DP, FP, and IP receptor expression by real-time polymerase chain reaction. (*C*) The effects of FP and IP receptor antagonists on Th17 cell differentiation of naive CD4$^+$ T cells were investigated. Naive CD4$^+$ T cells from wild-type mice were differentiated to Th17 cells in the presence or absence of the FP receptor antagonist, AL8810, the IP receptor antagonist, CAY10441, or a combination of AL8810 and CAY10441. The percentage of Th17 cells was analyzed by flow cytometry after 5 days. n = 3; * $P < 0.05$. (*D*) The effects of FP and IP receptor knockdown on Th17 differentiation of naive CD4$^+$ T cells were investigated. Naive CD4$^+$ T cells from wild-type mice were transfected with IP receptor siRNAs, FP receptor siRNAs, a combination of IP receptor and FP receptor siRNAs, or control siRNA. Transfected cells were then differentiated in the presence of anti-CD3 (3 μg/ml), anti-CD28 (3 μg/ml), anti–INF-γ (3 μg/ml), TGF-β (10 ng/ml), and IL-6 (10 ng/ml) for 5 days and the percentage of Th17 cells was analyzed by flow cytometry. n = 3; * $P < 0.05$ versus control siRNA.

processes (37, 38). Indeed, several recent studies suggest a possible role for PGE$_2$ in promoting T-cell differentiation. Activation of the EP4 receptor in T cells and dendritic cells not only facilitates Th1 cell differentiation but also amplifies IL-23–mediated Th17 cell expansion *in vitro* (19, 29). Administration of an EP4-selective antagonist *in vivo* decreases accumulation of both Th1 and Th17 cells in regional lymph nodes and suppresses disease progression in mice subjected to experimental autoimmune encephalomyelitis or contact hypersensitivity (39). *In vitro* differentiation of dendritic cells in the presence of PGE$_2$ alters the IL-12–IL-23 balance and promotes differentiation of Th17 cells via a paracrine mechanism (20). In purified human naive T cells, PGE$_2$ acts via EP2 and EP4 receptors to up-regulate IL-23 receptor and IL-1 receptor expression (19). Furthermore, PGE$_2$ acts in concert with IL-1β and IL-23 to drive RORγt, IL-17, IL-17F, CCL20, and CCR6 expression (29). Thus, previous studies suggest that PGE$_2$ promotes Th17 cell differentiation through an IL-23–IL-1 dependent paracrine pathway that involves dendritic cells (Figure 8A). Despite these studies, it is not known if COX-derived PGs other than PGE$_2$ are involved in Th17 cell differentiation, or if PGs directly influence Th17 cells during the differentiation process, independent of their effects on dendritic cells.

Th17 cell differentiation depends, at least in part, on the local cytokine microenvironment at the differentiation site (40). Key cytokines include TGF-β1, IL-1β, IL-2, IL-6, and IL-23, which are mainly produced by local macrophages, dendritic cells, and neutrophils. Consistent with the importance of locally produced cytokines in directing Th17 differentiation, we observed tissue-specific reductions in Th17 cells in lung and lymph nodes, but not in blood and spleen, of COX-2$^{-/-}$ mice after ovalbumin exposure. It is well known that COX-2 metabolites are involved in allergic lung inflammation (23, 41). PGE$_2$ affects antigen-presenting cell cytokine production, reducing the production of IL-12p35, IL-6, and tumor necrosis factor-α, and increasing the production of IL-10, IL-12p40, and IL-23 (42, 43). Thus, PGE$_2$ may impact the functional characteristics of T cells during priming. Proinflammatory cytokines, such as IL-1β and tumor necrosis factor-α, are dramatically up-regulated in the early phase of allergic lung inflammation and induce COX-2 in many cell types, including dendritic cells, macrophages, and T cells (43). COX-2 metabolites further enhance the secretion of cytokines in dendritic cells, macrophages, and T cells, including TGF-β1, IL-1β, IL-2, IL-6, IL-23, and also increase secretion of chemokines, which are critical for trafficking of other cell types to the site of allergic inflammation (43). Therefore, COX-2 products

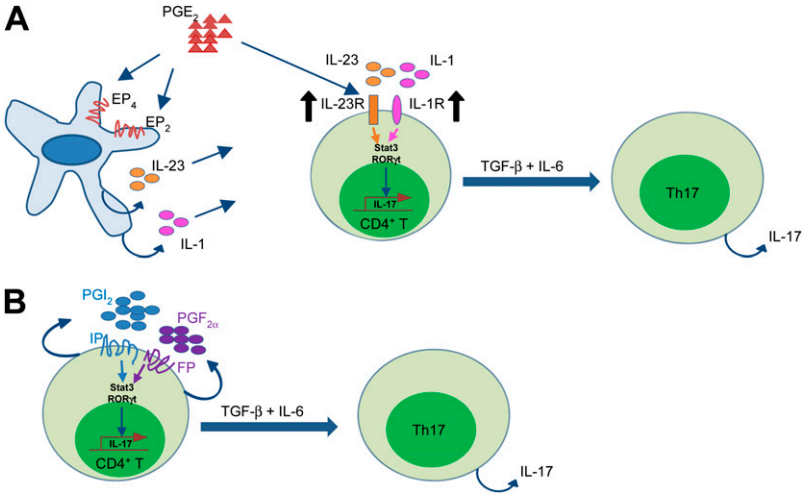

Figure 8. Proposed mechanisms for the regulation of Th17 cell differentiation by cyclooxygenase (COX)-2–derived prostaglandins (PGs). (*A*) Paracrine pathway of PGE_2 effects on Th17 cell differentiation. Binding of PGE_2 to EP2 and EP4 on dendritic cells leads to increased IL-23 and IL-1 expression. In addition, PGE_2 induces IL-23R and IL-1R expression on $CD4^+$ T cells. The IL-23 and IL-1 signals lead to activation of Stat3 and up-regulation of RORγt, which in conjunction with transforming growth factor (TGF)-β and IL-6, leads to IL-17 expression. (*B*) Autocrine pathway of PGI_2 and $PGF_{2\alpha}$ effects on Th17 cell differentiation. Binding of PGI_2 and $PGF_{2\alpha}$ to IP and FP receptors on $CD4^+$ T cells leads to activation of Stat3 and up-regulation of RORγt, which in conjunction with TGF-β and IL-6 leads to IL-17 expression.

regulate the cytokine and chemokine microenvironment during Th17 cell differentiation in a tissue-specific fashion.

COX-2 may directly regulate Th17 cell differentiation of naive $CD4^+$ T cells or may indirectly regulate Th17 cell differentiation by acting on other cell types. Several lines of evidence presented herein suggest the presence of a direct effect. First, we used highly purified naive $CD4^+$ $CD62L^+$ T cells from COX-$2^{+/+}$ and COX-$2^{-/-}$ mice for *in vitro* studies making confounding effects of other cell types unlikely. Second, we observed similar numbers of $CD4^+$ T cells and B cells in COX-$2^{+/+}$ and COX-$2^{-/-}$ mice suggesting that impaired T-cell development in COX-$2^{-/-}$ mice was not likely because of altered numbers of these cell types. Third, we observed up-regulation of COX-2 and enhanced PG biosynthesis in isolated $CD4^+$ T cells during Th17 cell differentiation *in vitro*. Fourth, we observed reduced levels of PGs in Th17 cell cultures from COX-$2^{-/-}$ mice. Finally, PGI_2 and $PGF_{2\alpha}$ were expressed in lung Th17 cells, and addition of $PGF_{2\alpha}$ and PGI_2 partially restored the Th17 differentiation defect of COX-$2^{-/-}$ cells *in vitro*. Thus, PGs that are produced by COX-2 in T cells act in an autocrine fashion during the differentiation process (Figure 8B). Previous studies may have overlooked the potent autocrine effects of COX-2–derived metabolites during T-cell differentiation. It should be noted that our data do not exclude the possibility that the reduced Th17 cell numbers in allergic COX-$2^{-/-}$ mice *in vivo* might be caused, at least in part, by effects of other cell types. In fact, our data showing that PGE_2 restores the Th17 differentiation defect *in vivo*, but not in isolated $CD4^+$ T cells *in vitro*, suggest an indirect effect of PGE_2 on cell types other than T cells, which in turn influences the Th17 cell differentiation process.

Others have observed PGE_2 promotion of Th17 cell differentiation *in vivo* through enhanced IL-23 secretion from dendritic cells (19, 29). In our *in vitro* experiments, PGE_2 did not restore the Th17 cell differentiation defect, whereas $PGF_{2\alpha}$ and PGI_2 enhanced Th17 cell differentiation of COX-$2^{-/-}$ naive $CD4^+$ T cells. To our knowledge, this is the first demonstration of an effect of $PGF_{2\alpha}$ and PGI_2 on Th17 cells during differentiation. The lack of an effect of PGE_2 in our *in vitro* experiments is likely caused by the exclusion of other cell types from this system. In the absence of dendritic cells, and the paracrine effects of PGE_2 on these cells, PGE_2 failed to restore the Th17 cell differentiation defect. Interestingly, the combination of PGI_2 and $PGF_{2\alpha}$ enhanced, but did not completely restore, Th17 differentiation and IL-17 production in COX-$2^{-/-}$ naive $CD4^+$ cells. Hence, it remains possible that other COX-2 metabolites, or a direct interaction of COX-2 with Stat3 or RORγt, may promote IL-17 production and Th17 cell differentiation (44, 45). In addition, differences in the percent of Th17 cells and IL-17 formation between COX-$2^{-/-}$ and WT mice persist despite PG treatment. This suggests that PG amplification may be secondary to the differential response of WT and COX-$2^{-/-}$ $CD4^+$ cells to the cytokine–antibody cocktail.

Consistent with our previous work demonstrating an inhibitory effect of PGs on Th2 immune responses (23), we found that lungs from allergic COX-$2^{-/-}$ mice showed increased airway inflammation compared with lungs from allergic COX-$2^{+/+}$ mice. Moreover, Th17 cells were primarily localized to sites of inflammation, which is consistent with the concept that Th17 cell differentiation is dependent on the local inflammatory cytokine milieu. Although there was increased airway inflammation in COX-$2^{-/-}$ mice after ovalbumin sensitization and exposure, fewer Th17 cells were found in lung inflammatory sites in COX-$2^{-/-}$ mice. One possible explanation for this apparent paradox is that allergic COX-$2^{-/-}$ mice also have increased numbers of Th2 cells (Figure E9) that are likely responsible, at least in part, for the enhanced allergic inflammatory response in the lungs of these mice.

Stat3 has been proposed to be a master regulator of Th17 cells, regulating Th17 cell differentiation and cytokine production, and induction of RORγt and IL-23R (46). IL-6 and TGF-β1 induce Th17 differentiation, whereas IL-23 is thought to be more important for *in vivo* maintenance of Th17 cells (31). Both IL-6 and IL-23 activate Stat3, a process that was found to be necessary for optimal Th17 differentiation. RORγt is a critical transcription factor in Th17 differentiation and is induced by the previously mentioned cytokines in a Stat3-dependent manner (47). PGE_2 activates the Stat3 signaling pathway to regulate tumor growth in a variety of human cancers through the EP1 receptor (48). PGI_2 induced Stat3 phosphorylation in human erythroleukemia cells (49). Our results demonstrate that Stat3 phosphorylation was lower in Th17 cells differentiated from naive $CD4^+$ T cells isolated from COX-$2^{-/-}$ mice. Stat3 activation by PGE_2 and PGD_2 was not observed in our studies, perhaps because of low DP, EP1, and EP4 receptor expression on $CD4^+$ T cells. However, PGI_2 and $PGF_{2\alpha}$ directly activated Stat3 phosphorylation during Th17 cell differentiation from naive $CD4^+$ T cells *in vitro*. Importantly, IP and FP receptor antagonists and siRNA knockdown of these receptors reduced Th17 cell differentiation of naive $CD4^+$ T cells *in vitro*. Together, these results provide compelling evidence that PGs bind to their cognate receptors to facilitate Th17 differentiation.

In summary, our findings demonstrate that COX-2 is a critical regulator of Th17 cell differentiation and function *in vitro* and *in*

vivo. Moreover, our results provide new mechanistic insights into how COX-2–derived PGs act to enhance Th17 cell differentiation of naive $CD4^+$ T cells. Future studies should examine the role of COX-derived eicosanoids in regulating the differentiation and function of other T-cell subsets.

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