Foxa2 Regulates Leukotrienes to Inhibit Th2-mediated Pulmonary Inflammation

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Foxa2 is a member of the Forkhead family of nuclear transcription factors that is highly expressed in respiratory epithelial cells of the developing and mature lung. Foxa2 is required for normal airway epithelial differentiation, and its deletion causes goblet-cell metaplasia and Th2-mediated pulmonary inflammation during postnatal development. Foxa2 expression is inhibited during aeroallergen sensitization and after stimulation with Th2 cytokines, when its loss is associated with goblet-cell metaplasia. Mechanisms by which *Foxa2* controls airway epithelial differentiation and Th2 immunity are incompletely known. During the first 2 weeks after birth, the loss of Foxa2 increases the production of leukotrienes (LTs) and Th2 cytokines in the lungs of Foxa2 gene–targeted mice. Foxa2 expression inhibited 15-lipoxygenase (Alox15) and increased Alox5 transcription, each encoding key lipoxygenases associated with asthma. The inhibition of the cysteinyl LT (CysLT) signaling pathway by montelukast inhibited IL-4, IL-5, eotaxin-2, and regulated upon activation normal T cell expressed and presumably secreted expression in the developing lungs of Foxa2 gene–targeted mice. Montelukast inhibited the expression of genes regulating mucus metaplasia, including *Spdef*, *Muc5ac*, *Foxa3*, and *Arg2*. Foxa2 plays a cell-autonomous role in the respiratory epithelium, and is required for the suppression of Th2 immunity and mucus metaplasia in the developing lung in a process determined in part by its regulation of the CysLT pathway.

Keywords: Foxa2; leukotriene; Th2 inflammation; mucous metaplasia

Goblet-cell metaplasia, mucus hyperproduction, and inflammation are associated with common chronic lung diseases, including asthma (1, 2). Evidence is increasing that the respiratory epithelium plays a role in the regulation of innate and acquired immunity in the setting of aeroallergen exposure (2). Foxa2, a member of the Forkhead family of transcription factors selectively expressed in respiratory epithelial cells, plays a critical role in suppressing Th2-mediated pulmonary inflammation and goblet-cell metaplasia in the developing murine lung (3). The expression of Foxa2 is sufficient to inhibit goblet-cell metaplasia after aeroallergen exposure in adult mice (3). Both Foxa2 and the thyroid transcription factor–1 (TTF-1) inhibit SAM pointed domain containing ETS transcription factor (SPDEF), which is an E–twenty six (Ets)-like transcription factor sufficient and necessary for the activation of genes regulating mucus production and goblet-cell metaplasia after aeroallergen or IL-13 exposure (3, 4).

Lipid-derived mediators play an important role in lung inflammation (5). Proinflammatory lipid mediators derived from arachidonic acid, including leukotrienes (LTs), are the metabolic products of arachidonic acid, and are considered important inflammatory mediators during asthma (6). LTs are synthesized through multiple enzymatic steps from membrane phospholipids. Arachidonic acid is cleaved from membrane phospholipids via phospholipase A2. Arachidonic acid is converted to 5(S)-hydroperoxy-6-trans-8,11,14-cis-eicosate-traenoic acid and then to leukotriene A4 (LTA4) in two oxidative steps by 5-lipoxygenase (Alox5). The Alox5-activating protein is necessary for this conversion. LTA4 is conjugated with the tripeptide glutathione to form the first of the cysteinyl-LTs LTC4. Alternatively, LTA4 can be metabolized to the hydroxyl LTB4 by epoxide hydrolase in neutrophils and other inflammatory cells. LTB4 is a potent neutrophil and eosinophil chemoattractant. LTC4 is exported to the extracellular space and cleaved to LTD4 and LTE4. LTC4, LTD4, and LTE4 all contain a cysteinyl residue and are collectively called the CysLTs (5). LT antagonists, mainly antagonists of the $CysLT_1$ receptor, are used in clinical practice for the treatment of asthma (7). LT responses are mediated through the activation of the cell surface–specific, G-protein–coupled receptors leukotriene B4 receptor 1 (BLT1) and BLT2 for LTB4 signaling, and $CysLT_1$ and $CysLT_2$ for CysLT signaling (8, 9). $CysLT_1$ signaling is blocked by specific antagonists, including montelukast, zafirlukast, and pranlukast, which do not target $CysLT_2$ (7). IL-4, IL-5, and IL-13 augment CysLT production. IL-4 up-regulates LTC4 synthase and $CysLT_1$ receptor gene expression (10). LT synthase is induced by IL-13 in human lung macrophages (11). In *Ltc4 s*^{null} mice, the production of IL-4, IL-5, and IL-13 by antigen restimulation was reduced (12). IL-13 enhances the expression of the $CysLT_1$ receptor in human lung fibroblasts, increasing eotaxin production in a concentration-dependent fashion (13). How the Th2 cytokine and LT pathways interact during Th2-mediated pulmonary inflammation remains poorly understood.

The role of the LT pathway in Th2-mediated pulmonary inflammation caused by Foxa2 deletion in airway epithelial cells remains unknown. In this study, we hypothesized that Foxa2 regulates the LT pathway to suppress Th2-mediated inflammation in the lung. The present data demonstrate that Foxa2 directly regulated *Alox15* and *Alox5* gene transcription. Montelukast, a selective antagonist of the $CysLT_1$ receptor, inhibited the Th2-mediated inflammation and mucus metaplasia caused by the deletion of Foxa2 in the developing mouse lung. Activation of the LT pathway in developing *Foxa2* gene–targeted mice preceded the increased expression of Th2 cytokines. Taken together, Foxa2 is required for the suppression of Th2-mediated pulmonary inflammation and mucus metaplasia during lung development in a process determined in part by its regulation of the CysLT pathway.

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MATERIALS AND METHODS

More detailed methods are available in the online supplement.

Transgenic Mice and Animal Husbandry

Mice were maintained under pathogen-free conditions according to protocols approved by the Institutional Animal Care and Use Committee of West China Hospital at Sichuan University. *Foxa2*$^{loxP/loxP}$ mice were kindly provided by Dr. Klaus Kaestner at the University of Pennsylvania (Philadelphia, PA). The conditional deletion of Foxa2 in airway epithelial cells was performed as previously described, using the *SPC*-rtTA-tet(o)7-CRE system (14). Mice were killed by an injection of anesthetic, and exsanguinated. Foxa2 deletion was confirmed by quantitative PCR.

Montelukast Treatment

Intraperitoneal injections were performed in neonatal Surfactant protein C (*SFTPC*)/*Foxa2*$^{\Delta/\Delta}$ pups and control littermates with either montelukast (10 mg/kg body weight; a gift from Merck Sharp and Dohme Corporation, Rahway, NJ) or the corresponding volume of sterile saline daily from Postnatal Day (PN) 5 to PN15. On PN15, bronchoalveolar lavage fluid (BALF) was collected and differential cell counts were measured, as previously described (3). The left lungs and tracheas were fixed in 4% paraformaldehyde and processed into paraffin blocks for hematoxylin and eosin staining. Lung tissue was collected and stored at -70°C for quantitative PCR, Western blotting, or ELISA.

Measurement of Cytokines and Mediators in the BALF and Lung

Lung tissue from PN5, PN7, and PN15 were prepared. The levels of cytokines (IL-13, IL-4, IL-5, TNF-α, and IL-1β), normal T cells expressed and secreted (RANTES), eotaxin-2, the thymus and activation–regulated chemokine, and LTB4, LTC4, LTD4, LTE4, 12-hydroxy-eicosatetraenoic acid (12-HETE), prostaglandin H_2 (PGH2), and prostaglandin E_2 (PGE2) in the BALF supernatant and in the supernatant of lung homogenates were measured with ELISA kits, according to the manufacturer's protocol. Mouse ELISA kits were purchased from Cusabio Biotech Co., Ltd. (Wuhan, Hubei, China).

Western Blot Analysis

Western blot analysis was performed on lung homogenates from *SFTPC-Foxa2*$^{\Delta/\Delta}$ mice and control littermates at PN5 and PN15. The protein was separated by SDS-PAGE, transferred, and immobilized on nitrocellulose membranes. Membranes were incubated with goat anti-mouse Alox15 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA) or the $CysLT_1$ receptor (1:200; Santa Cruz Biotechnology) antibody.

Plasmid Construction and Luciferase Reporter Assay

Alox15 and Alox5 promoter–luciferase constructs were constructed with pGL3 plasmids. The constructs were transiently transfected into H441 cells, and luciferase activity was assessed 48 hours after the transfection.

RNA Extraction and Quantitative Real-Time RT-PCR

Total RNA from lung tissue was extracted with Trizol (Life Technologies, Grand Island, NY). Reverse transcriptase and quantitative real-time PCR analyses were performed.

RNA Microarray Analysis

Lung cDNA was hybridized to murine genome MOE430 chips (Affymetrix, Santa Clara, CA), according to the manufacturer's protocol, and data processing was performed as previously reported (14). The data were derived from a previous study (3).

Splenocyte Activation and Cytokine Assay

Splenocytes from *SPC-Foxa2*$^{\Delta/\Delta}$ mice and control littermates were constructed, cultured, and stimulated with anti-CD3 and anti-CD28 (Pharmingen, San Diego, CA), and then IL-13, IL-4, and IFN-γ were measured.

Statistical Analysis

Analyses were performed using SPSS, version 13.0 (SPSS, Inc., Chicago, IL). Data are expressed as means ± SEMs. ANOVA, the Tukey-Kramer test, the Dunnett test, and the unpaired *t* test were used to compare differences in groups. Differences were considered statistically significant at $P < 0.05$, according to a two-tailed test.

RESULTS

Conditional Deletion of Foxa2 Induced Th2 Pulmonary Inflammation

Foxa2 was conditionally deleted from respiratory epithelial cells under the control of *SFTPC*-rtta, tet(o)7-CRE to mediate the selective deletion of the floxed *Foxa2* gene in the epithelia of developing mouse lungs. In whole lungs, *Foxa2* mRNA was decreased approximately twofold when assessed on PN 15 (Figure 1A). Extensive pulmonary inflammation was observed in the lungs of *Foxa2*$^{\Delta/\Delta}$ mice, and this finding was supported by the quantification of inflammatory cells in BALF, wherein eosinophils, macrophages, lymphocytes, and neutrophils were increased (Figure 1B). Consistent with previous findings (3), the deletion of Foxa2 was associated with the increased expression of the Th2 cytokines IL-13 and IL-4 (Figure 1C). However, Th1 cytokines, such as IL-1β and TNF-α, were similar in both groups (Figure E1 in the online supplement).

Components of the LT Signaling Pathways Are Activated after Foxa2 Deletion in Airway Epithelial Cells

To investigate the role of Foxa2 and its downstream targets known to be associated with Th2 inflammation, an mRNA microarray (3) was used to assess differential gene expression in lung tissue from control and *Foxa2*-deleted mice. The mRNAs encoding LT signaling pathway–related enzymes (Alox5, 5-lipoxygenase-activating protein [FLAP], and LTC4 s) and Alox15 were significantly increased in lungs from *Foxa2*$^{\Delta/\Delta}$ mice, findings confirmed by quantitative RT-PCR. LTB4, LTC4, LTD4, LTE4, and 12-HETE were increased in lung-tissue homogenates from *Foxa2*$^{\Delta/\Delta}$ mice, as determined by ELISA assay. These data indicate that the LTs and other components of the lipoxygenase network are activated during the Th2 lung inflammation caused by Foxa2 deletion in airway epithelial cells during postnatal development (Figure 2). We also measured PGH2 and PGE2, the products of the cyclooxygenase-2 (COX-2) pathway, which plays an important role in some inflammation. However, neither PGH2 nor PGE2 changed in lung-tissue homogenates from *Foxa2*$^{\Delta/\Delta}$ and control mice (Figure E2), indicating that Foxa2 deletion did not activate the COX-2 pathway.

Activation of LT Components Was Independent of Th2 Cytokines in *Foxa2*$^{\Delta/\Delta}$ Mice

The expression of mRNAs encoding components of the lipoxygenase signaling pathway and Th2 cytokines was increased in the lungs of *Foxa2*$^{\Delta/\Delta}$ mice. To determine the timing of Th2 cytokines and lipid-derived mediators influenced by Foxa2 deletion, levels of LTB4, LTC4, LTD4, LTE4, 12-HETE and the Th2 cytokines IL-4, IL-5, and IL-13 were assessed on PN5 and PN7 by ELISA. The mRNAs of Th2 cytokines and enzymes related to lipid-derived mediator metabolism were also measured by quantitative RT-PCR at the same time. LTB4, LTD4, LTE4, and 12-HETE were significantly increased in lung homogenates from *Foxa2*$^{\Delta/\Delta}$ mice as early as PN5, at a time when the expression

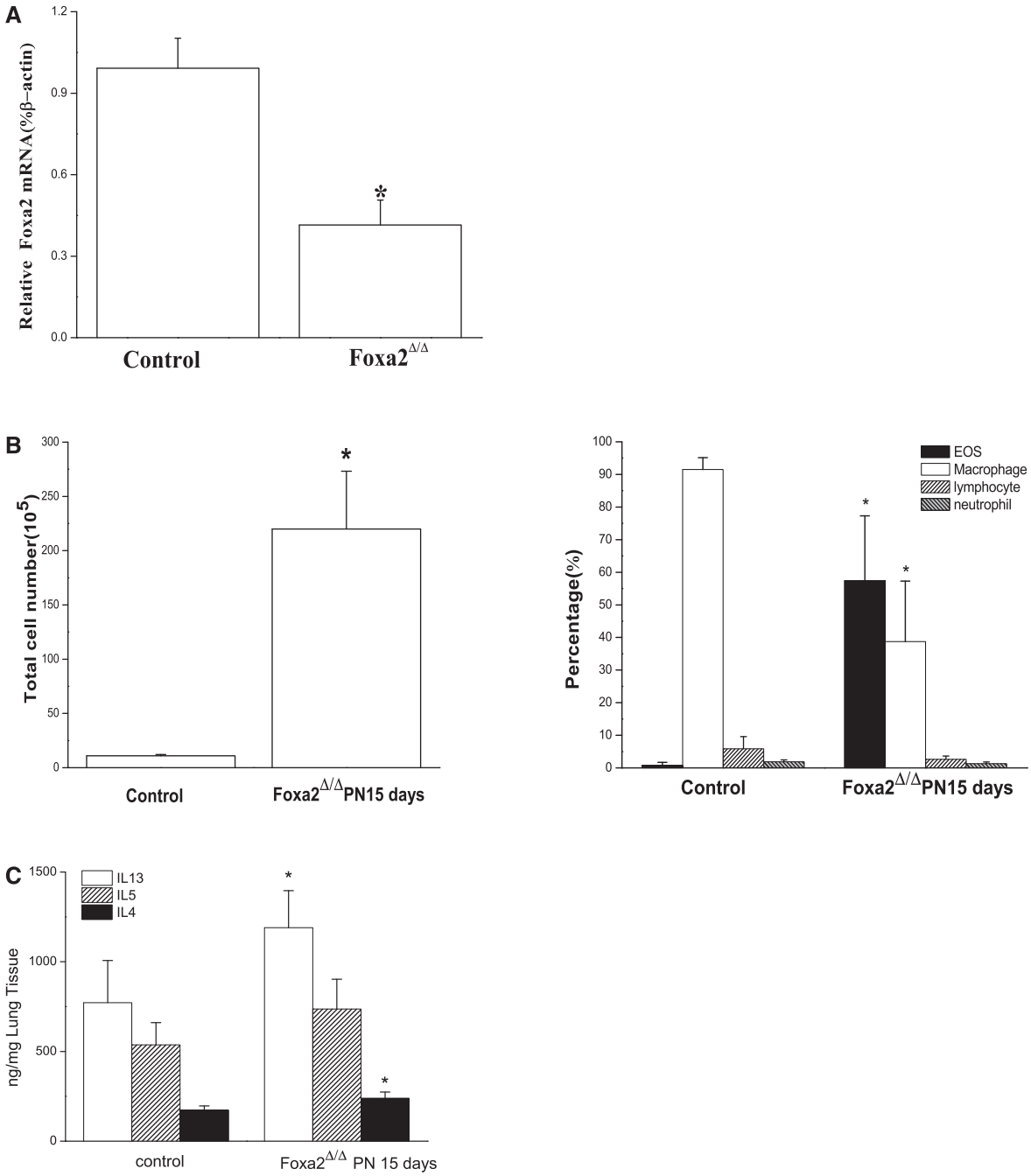


Figure 1. Deletion of *Foxa2* in respiratory epithelium caused pulmonary eosinophilic inflammation on Postnatal Day (PN) 15. (*A*) Loss of Foxa2 expression was confirmed by RT-PCR analysis. *Foxa2* mRNA was significantly decreased. (*B*) Numbers of total cells and eosinophils (EOS) in bronchoalveolar lavage fluid (BALF) were increased in *Foxa2*$^{\Delta/\Delta}$ mice on PN15. Eosinophils in BALF were increased, as assessed by Diff-Quik (Polysciences, Warrington, PA) staining. (C) Th2 cytokines (IL-4, IL-5, and IL-13) were increased in the lung tissue of *Foxa2*$^{\Delta/\Delta}$ mice, as determined by ELISA. The graphs represent means ± SEs ($n = 5$ for each genotype). *$P < 0.05$ versus control values, using the Student *t* test.

of IL-4 and IL-13 was similar in control and *Foxa2*$^{\Delta/\Delta}$ mice (Figure 3A). Alox5 and LTC4 s mRNAs were increased on PN5 (Figure 3B). On PN7, Alox5, Alox15, FLAP, and LTC4 s mRNAs were increased in *Foxa2*$^{\Delta/\Delta}$ mice (Figure 3D), and LTB4 and LTE4 concentrations in lung homogenates were higher in *Foxa2*$^{\Delta/\Delta}$ mice than in control mice (Figure 3C). IL-4, IL-5, and IL-13 mRNAs were increased, but not to a statistically significant extent (Figure 3D). In contrast, IL-4, IL-5, and eotaxin-2 protein levels were higher in *Foxa2*$^{\Delta/\Delta}$ mice than in control mice (Figure 3C). The $CysLT_1$ receptor was increased in lungs of *Foxa2*$^{\Delta/\Delta}$ mice on PN5 (Figure 3E), as assessed by Western blotting.

Foxa2 Inhibited the Transcription of *Alox15*, but Increased the Transcription of the *Alox5* Gene *In Vitro*

Alox15 plays a key role in Th2 inflammation in the lung (15). Alox15 mRNA was significantly increased in the lungs of *Foxa2*$^{\Delta/\Delta}$ mice. To determine whether Foxa2 directly regulated Alox15 and Alox5 in respiratory epithelial cells, luciferase reporter constructs

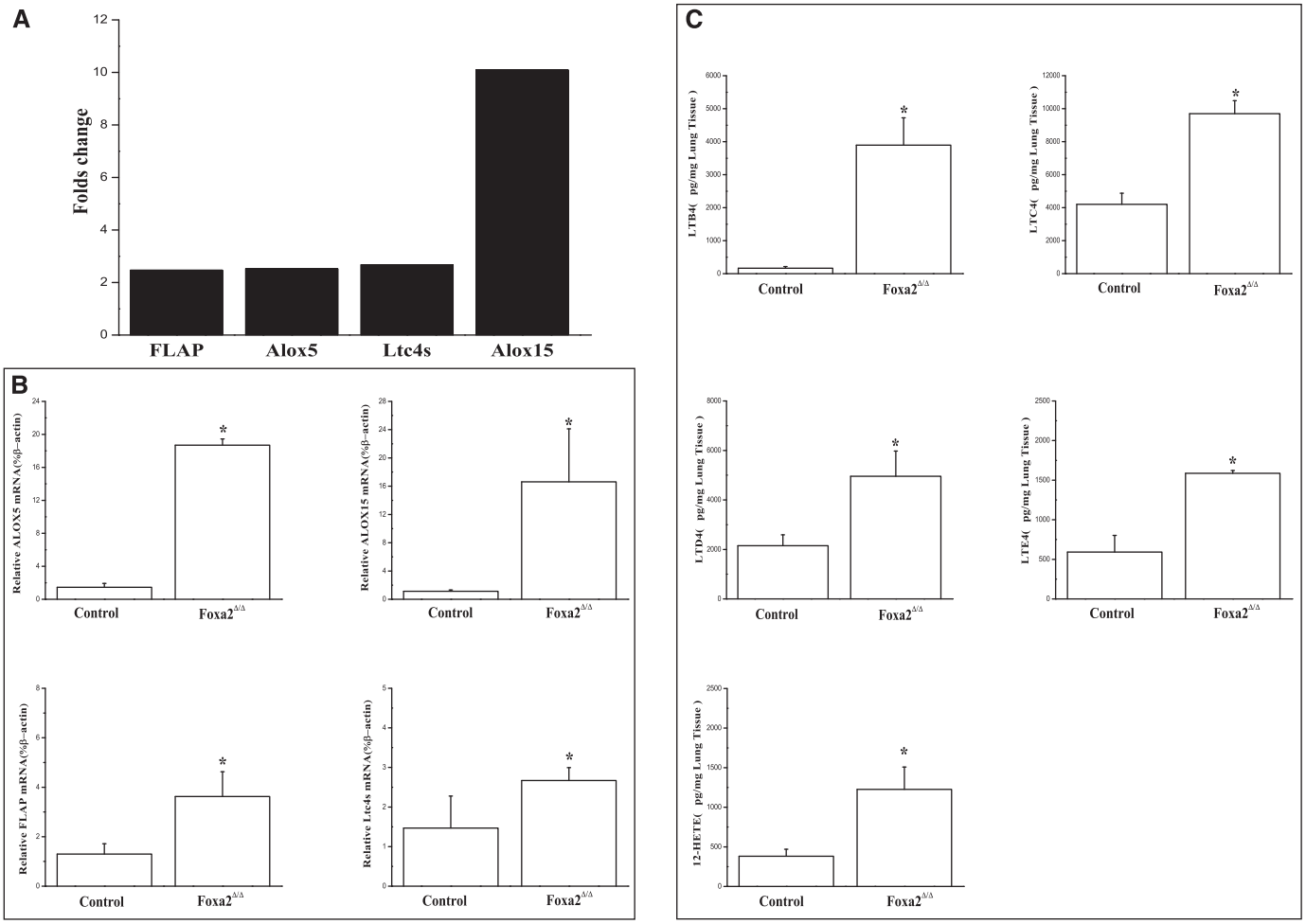


Figure 2. Leukotriene (LT) pathways are involved in the Th2 lung inflammation caused by Foxa2 deletion in airway epithelial cells. (*A*) cDNAs were constructed from *Foxa2*$^{\Delta/\Delta}$ mice and control littermates on PN15 ($n = 3$ of each genotype), and hybridized to murine genome MOE430 chips (Affymetrix). Microarray analysis showed that the loss of Foxa2 in respiratory epithelial cells significantly up-regulated mRNAs encoding arachidonic acid metabolism–related enzymes, including 5-lipoxygenase (Alox5), Alox15, FLAP, and LTC4 s. (*B*) Increased LT-related enzyme and Alox15 mRNAs in *Foxa2*$^{\Delta/\Delta}$ mice on PN15 ($n = 5$ of each genotype) are shown. Quantitative RT-PCR results were consistent with mRNA microarrays. (*C*) LTB4, LTC4, LTD4, LTE4 and 12-HETE were increased in *Foxa2*$^{\Delta/\Delta}$ mice on PN15, as detected by ELISA. Graphs represent means ± SEs ($n = 5$ for each genotype for ELISA). *$P < 0.05$, versus control values determined by the Student *t* test.

containing regulatory regions of the mouse *Alox15* and *Alox5* genes were cotransfected into H441 cells with Foxa2 expression plasmids. Transfected cells were further stimulated with IL-13, known to induce the expression of Alox15 (15, 16). Foxa2 significantly inhibited the transcription of *Alox15* (Figure 4A). IL-13 exerted no effect on the activity of the Alox15 promoter, with or without Foxa2. In contrast, Foxa2 increased Alox5 promoter activity in a dose-dependent manner. This result indicated that Foxa2 differentially regulated the activity of the *Alox15* and *Alox5* genes (Figures 4B and 4C) in this cell line. The stimulatory effect of Foxa2 on Alox5 promoter activity contrasts with the finding that Alox5 mRNA was increased in the Foxa2-deleted mice.

Splenocytes Were Not Activated in Th2-Mediated Pulmonary Inflammation after the Deletion of *Foxa2*

In the present study, Foxa2 was selectively deleted in respiratory epithelial cells. To assess whether the loss of Foxa2 influenced systemic immunity, spleen cells were isolated from *Foxa2*$^{\Delta/\Delta}$ and control mice on PN15 and activated by anti-CD3 and anti-CD28. IL-13, IL-4, and IFN-γ were measured by ELISA in supernatants from activated or inactivated splenocytes. IL-4, IL-13, and IFN-γ concentrations were higher in supernatants from activated splenocytes than inactivated splenocytes, but they were not different in *Foxa2*$^{\Delta/\Delta}$ and control mice (Figure 5). These findings demonstrate that Th2-mediated inflammation seen after deletion of Foxa2 was likely confined to the lung.

Pulmonary Eosinophilic Inflammation Induced by Foxa2 Deletion Is Inhibited by Montelukast

CysLT signaling is a key pathway regulating Th2 inflammation in asthma. Montelukast inhibits eosinophil adhesion via several $CysLT_1$ receptor–dependent mechanisms (17, 18). To investigate whether pulmonary inflammation caused by the conditional deletion of *Foxa2* in the airway epithelium was associated with activation of the CysLT pathway, Foxa2$^{\Delta/\Delta}$ mice were treated with montelukast daily from PN5–PN15. Inhibition of the CysLT signaling pathway was sufficient to inhibit Th2 inflammation, as indicated by histopathology and the inhibition of expression of Th2 cytokines and chemokines in the lung (Figures 6A and 6E). Montelukast treatment inhibited pulmonary inflammation, as also indicated by the reduction of BALF cells ($P < 0.05$; Figure 6B). LT production, including LTB4, LTC4, LTD4, LTE4, and

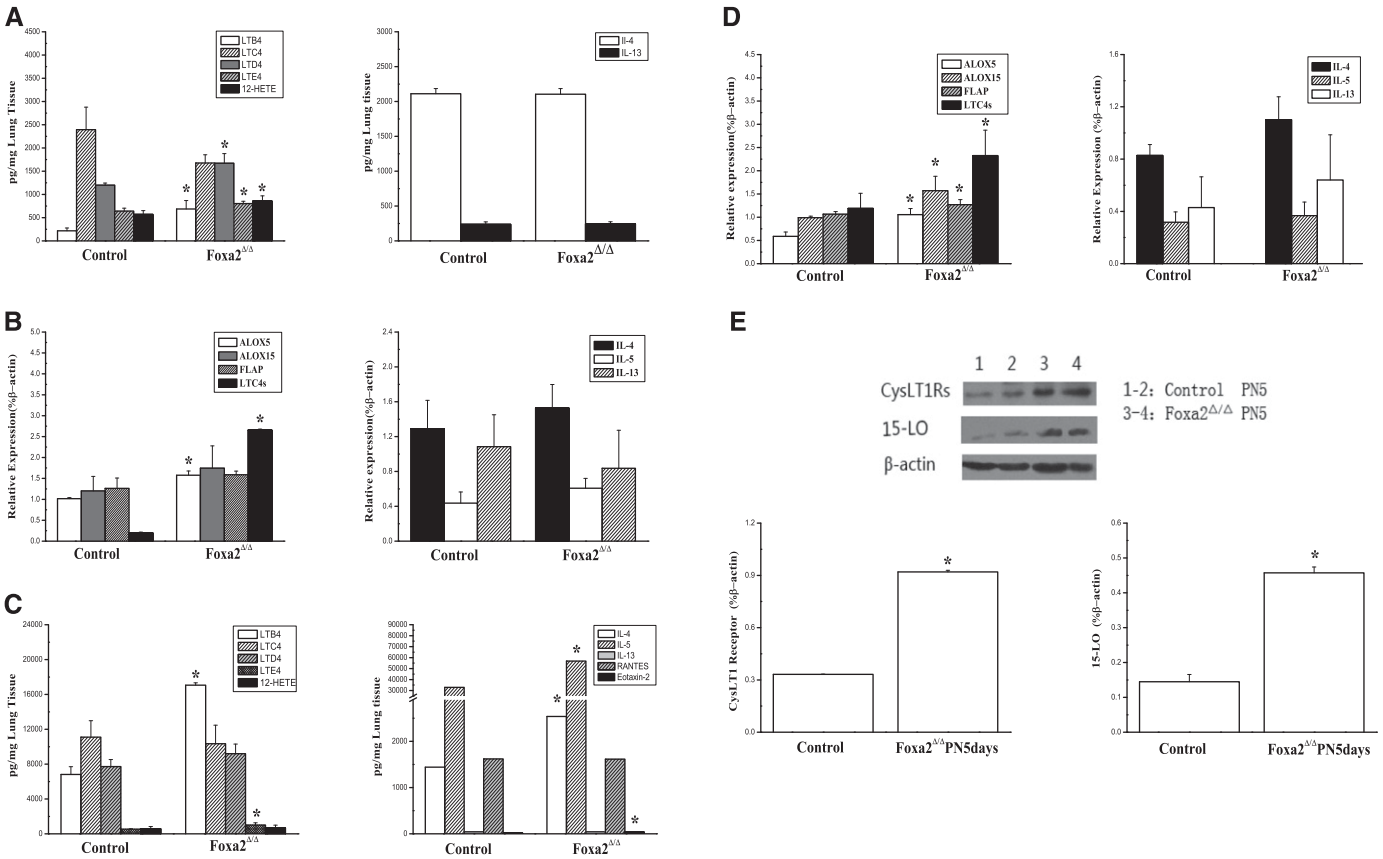


Figure 3. Th2 cytokines, LTs, and 12-HETE in *Foxa2*$^{\Delta/\Delta}$ mice. (*A*) ELISA assay results revealed increased LTs and 12-HETE in *Foxa2*$^{\Delta/\Delta}$ mice on PN5, but with no difference in protein levels of IL-4, IL-5, and IL-13 in control and *Foxa2*$^{\Delta/\Delta}$ mice. (*B*) Quantitative RT-PCR results indicated that the expression of LT-related enzymes increased in *Foxa2*$^{\Delta/\Delta}$ mice, but with no difference in Th2 cytokine mRNA. (*C*) Th2 cytokines, LTs, and 12-HETE both exhibited a trend to increase significantly, compared with the control group, according to ELISA on PN7. (*D*) The mRNAs of LT-related enzymes and Th2 cytokines exhibited a trend to increase in *Foxa2*$^{\Delta/\Delta}$ mice, measured using quantitative RT-PCR on PN7. (*E*) Western blots of Alox15 (15-LO), the cysteinyl–leukotriene–1 (CysLT$_1$) receptor, and β-actin in lungs from *Foxa2*$^{\Delta/\Delta}$ mice on PN5. The graph data *below* were derived from three different experiments. *$P < 0.05$, versus control values determined by two-tailed Student *t* test ($n = 5$ for each genotype).

the production of Alox15 and 12-HETE were inhibited by montelukast (Figures 6C and 6D), and IL-4, IL-5, RANTES, and eotaxin-2 were significantly decreased. In contrast, levels of IL-13 remained higher, compared with control samples (Figure 6E). Western blot analysis indicated that Alox15 and CysLT$_1$ receptor expression was decreased by montelukast (Figure 6F). These results indicate that Th2 inflammation induced by the deletion of *Foxa2* was partly dependent upon the CysLT signaling pathway.

Goblet-Cell Metaplasia Induced by Foxa2 Deletion Was Inhibited by Montelukast

Because the LT pathway is thought to play a role in goblet-cell metaplasia (19), markers associated with the production of airway mucus were measured after montelukast administration. *Spdef*, *Muc5ac*, *Arg2*, and *Foxa3* mRNAs were significantly decreased by montelukast (Figure 7). These results indicate that the goblet-cell metaplasia induced by Foxa2 deletion during neonatal lung development is, at least in part, regulated by the LT pathway.

DISCUSSION

Lung formation and epithelial-cell differentiation are regulated by a complex network of transcription factors, in which TTF-1, GATA-6, and the Forkhead transcription factors FOXA1, FOXA2, FOXF1, and FOXJ1 play important roles (20). Functional analyses of the regulatory regions of several lung-specific genes demonstrated the important role of Foxa2 in regulating the transcription of genes that influence lung morphogenesis and homeostasis (21, 22). The deletion of *Foxa2* in respiratory epithelial cells caused spontaneous pulmonary Th2 inflammation after birth, in association with the increased expression of Th2 cell–associated cytokines and chemokines (3). Moreover, the conditional expression of Foxa2 inhibited goblet-cell metaplasia during allergen sensitization in adult mice (3). In the present study, we found that the production of CysLTs and Alox15 were increased during spontaneous pulmonary Th2 inflammation after the deletion of Foxa2. The LT pathway was activated as early as PN5 in the lungs of *Foxa2* gene–deleted mice. the activation of LTs and other components of the lipoxygenase pathway preceded the increased expression of the Th2 cytokines, IL-4, IL-5, and IL-13, during development. Foxa2 directly regulated the transcription of Alox15 and Alox5, key enzymes for lipid-derived mediators. Inhibition of the CysLT signaling pathway attenuated the Th2 airway inflammation induced by Foxa2 deletion, without affecting IL-13. Lastly, splenocytes were not activated in the Th2-mediated pulmonary inflammation caused by Foxa2 deletion. Our results indicate that Foxa2 is required for the inhibition of the LT signaling pathway in the developing lung, and that spontaneous activation

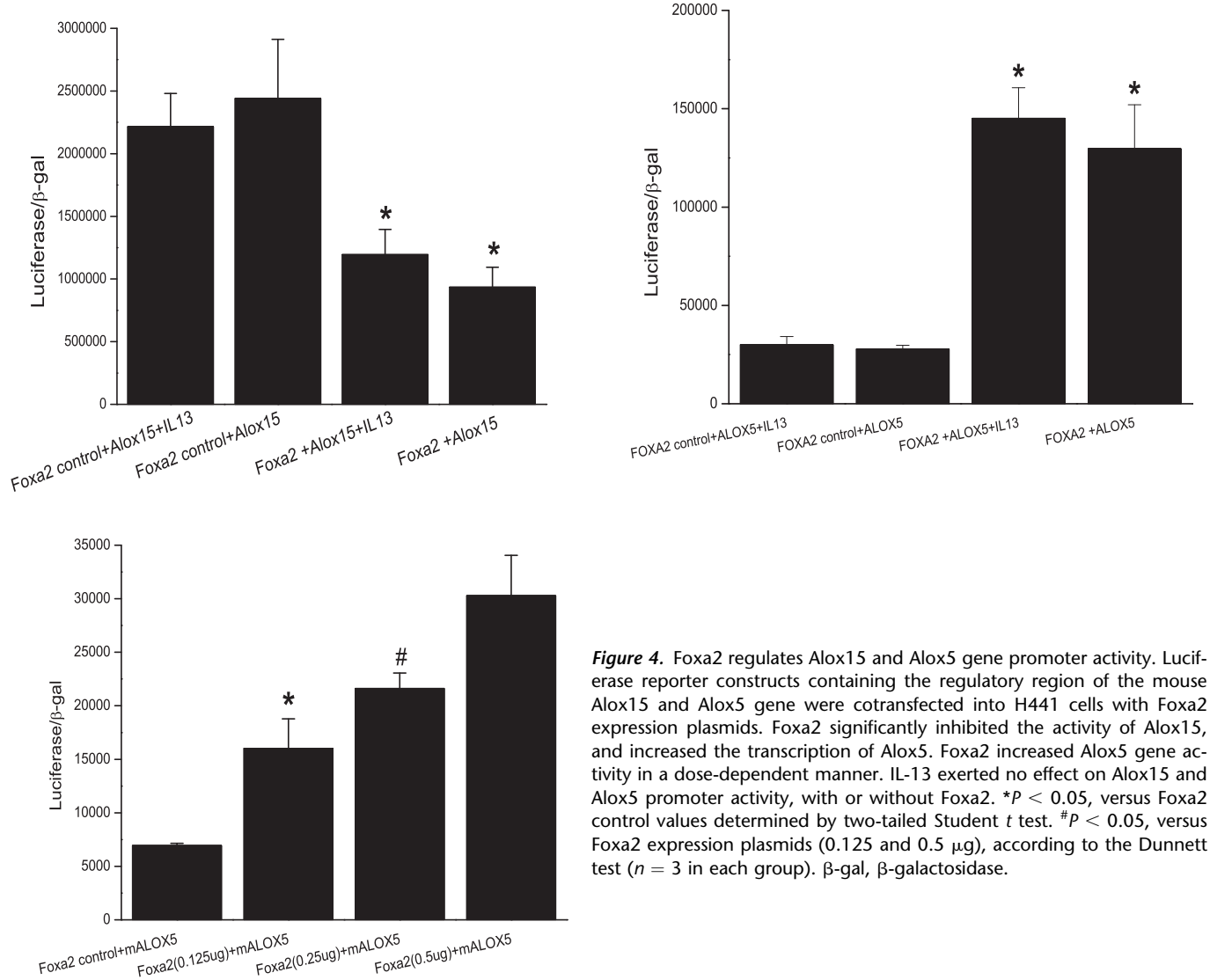


Figure 4. Foxa2 regulates Alox15 and Alox5 gene promoter activity. Luciferase reporter constructs containing the regulatory region of the mouse Alox15 and Alox5 gene were cotransfected into H441 cells with Foxa2 expression plasmids. Foxa2 significantly inhibited the activity of Alox15, and increased the transcription of Alox5. Foxa2 increased Alox5 gene activity in a dose-dependent manner. IL-13 exerted no effect on Alox15 and Alox5 promoter activity, with or without Foxa2. $^{*}P < 0.05$, versus Foxa2 control values determined by two-tailed Student *t* test. $^{\#}P < 0.05$, versus Foxa2 expression plasmids (0.125 and 0.5 μg), according to the Dunnett test ($n = 3$ in each group). β-gal, β-galactosidase.

of the LT pathway is involved in the Th2-mediated pulmonary inflammation resulting from *Foxa2* gene deletion in the airway epithelium.

The LT pathway plays an important role in asthma Th2-mediated pulmonary inflammation. Our study demonstrated that the deletion of Foxa2 in airway epithelium activated the LT pathway in the lung, as indicated by the increased expression of Alox5, FLAP, and LTC4 s mRNAs on PN15. The increased expression of Alox15 and the production of 12-HETE indicate that other components of the lipoxygenase pathway were involved in the Th2-mediated pulmonary inflammation caused by Foxa2 deletion. Activation of the lipoxygenase pathway occurred as early as PN5, before the increased expression of Th2 cytokines. *Alox15* promoter activity was inhibited, whereas the *Alox5* promoter was

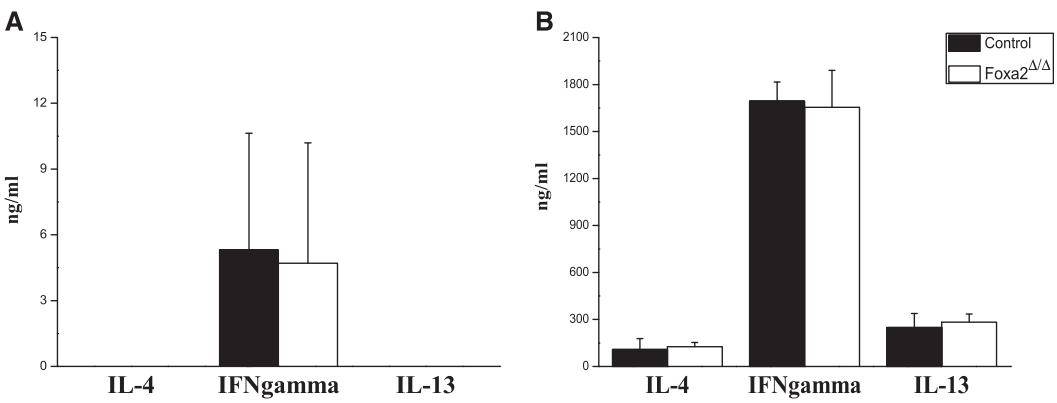
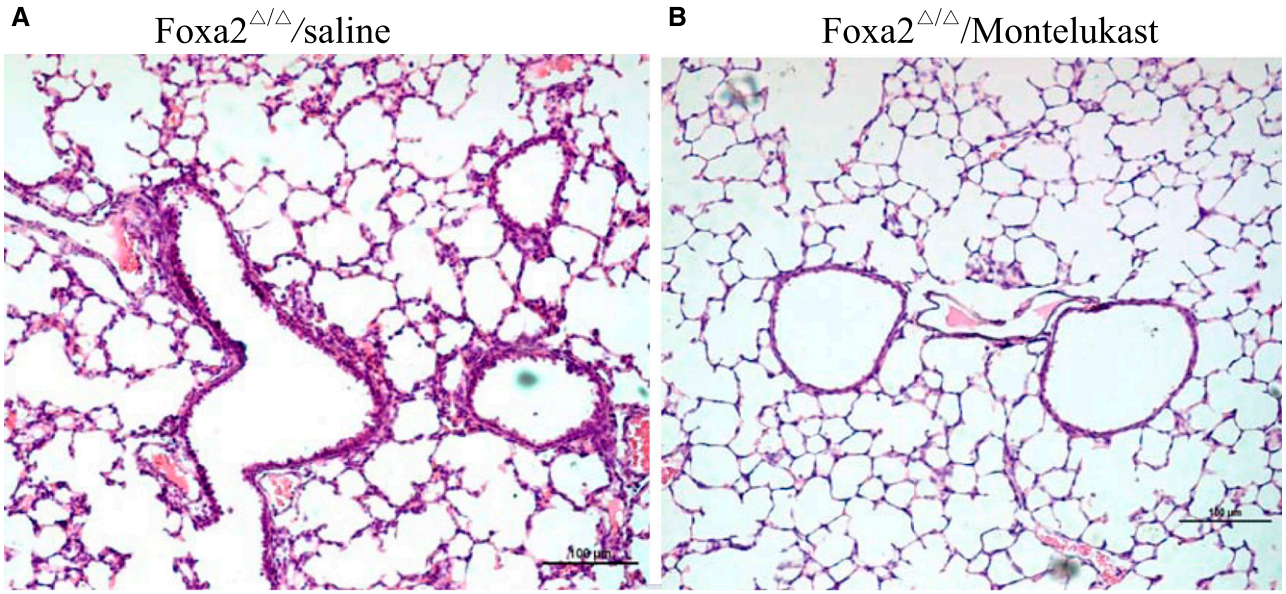


Figure 5. Cytokines produced by splenocytes from *Foxa2*$^{\Delta/\Delta}$ and control mice. (*A*) Splenocytes from *Foxa2*$^{\Delta/\Delta}$ and control mice on PN15, without anti-CD3 and anti-CD28. (*B*) Splenocytes from *Foxa2*$^{\Delta/\Delta}$ and control mice on PN15 were stimulated with anti-CD3 and anti-CD28. The concentrations of IL-13, IL-4, and IFN-γ were measured by ELISA in the supernatants. $^{*}P < 0.05$, versus control values determined by Student *t* test (two-tailed; $n = 3$ for each genotype).



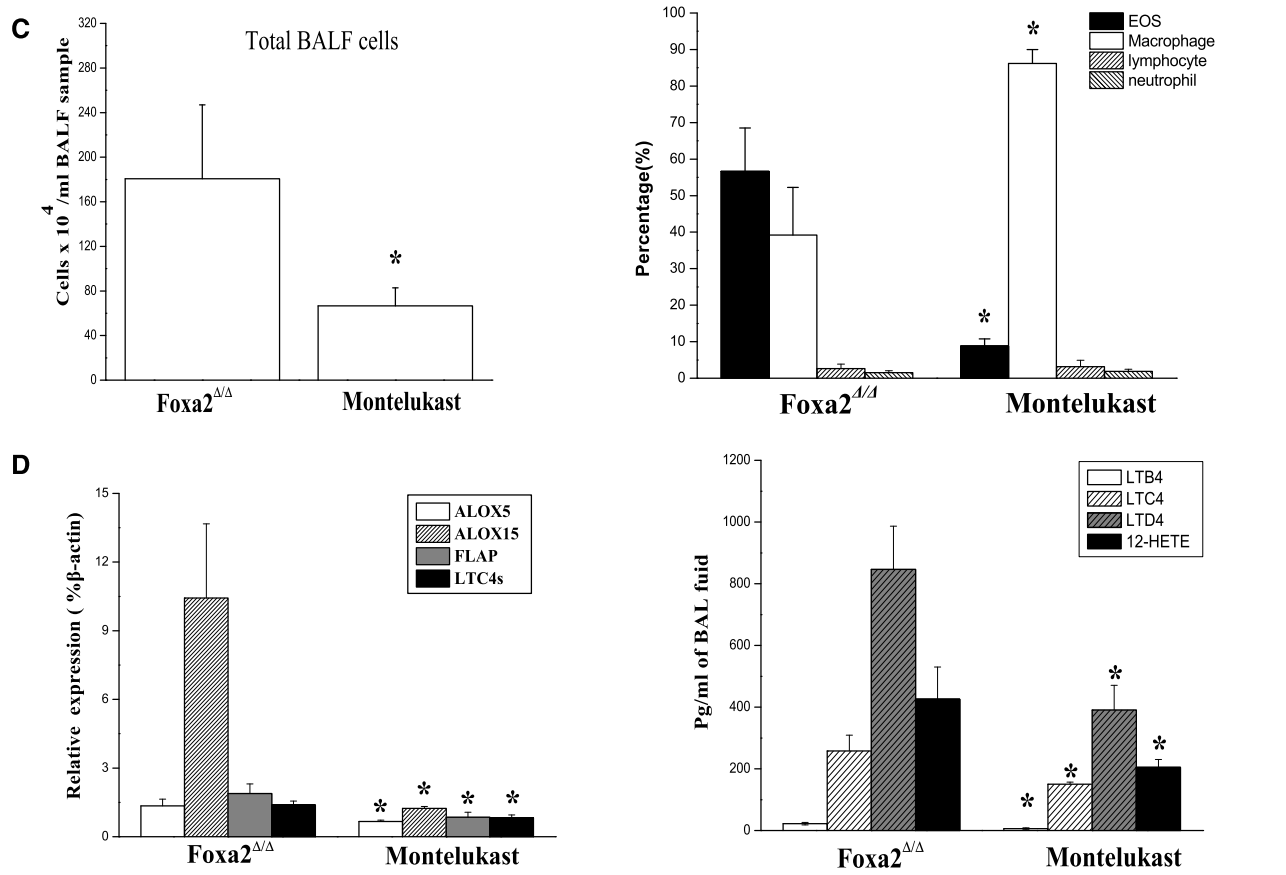
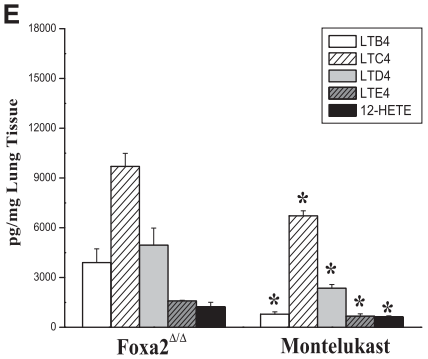
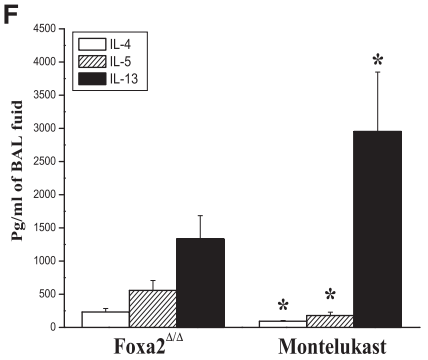
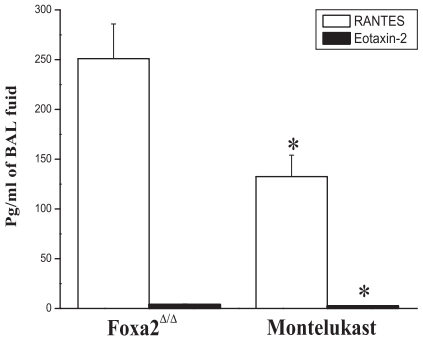
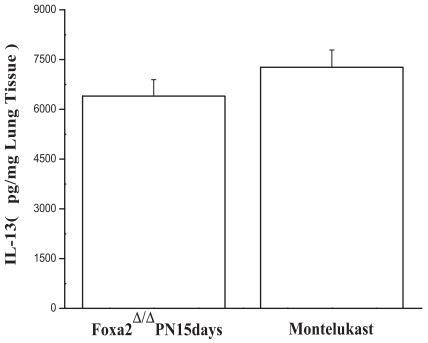


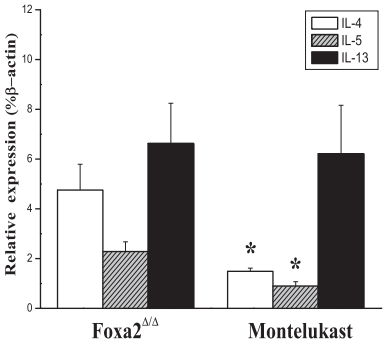
Figure 6. Montelukast attenuated Th2 lung inflammation in *Foxa2*$^{\Delta/\Delta}$ mice. Neonatal *SFTPC/Foxa2*$^{\Delta/\Delta}$ pups and control littermates were injected with either montelukast or sterile saline from PN5–PN15. (*A* and *B*) Hematoxylin and eosin staining of lung tissues from *Foxa2*$^{\Delta/\Delta}$ mice with saline or montelukast. (*C*) Numbers of cells in BALF were assayed. Decreased numbers of total cells and eosinophils were found in BALF from *Foxa2*$^{\Delta/\Delta}$ mice after montelukast treatment. (*D*) Lung RNAs were extracted from *Foxa2*$^{\Delta/\Delta}$ and *Foxa2*$^{\Delta/\Delta}$ mice with montelukast on PN15. Alox5, Alox15, FLAP, and LTC4 s mRNAs were decreased in *Foxa2*$^{\Delta/\Delta}$ mice after montelukast treatment on PN15, compared with those mRNAs in the control group according to RT-PCR. (*E*) LTB4, LTC4, LTD4, LTE4, and 12-HETE were decreased in BALF and lung homogenates compared with control samples, according to ELISA. (*F*) BALF and lung homogenate levels of Th2 cytokines and chemokines, including IL-4, IL-5, RANTES, and eotaxin-2, were decreased in *Foxa2*$^{\Delta/\Delta}$ mice after montelukast treatment, compared with control mice. (*G*) Western blots of Alox15, cysLT1 receptor, and β-actin in lungs from *Foxa2*$^{\Delta/\Delta}$ mice after montelukast or saline treatment on PN15. The graph data *below* were derived from three different experiments. Graph represents means ± SEMs ($n = 5$ for each genotype) for quantitative RT-PCR and ELISA assays. *$P < 0.05$, versus control values determined by the Student *t* test (two-tailed; $n = 5$ of each genotype).

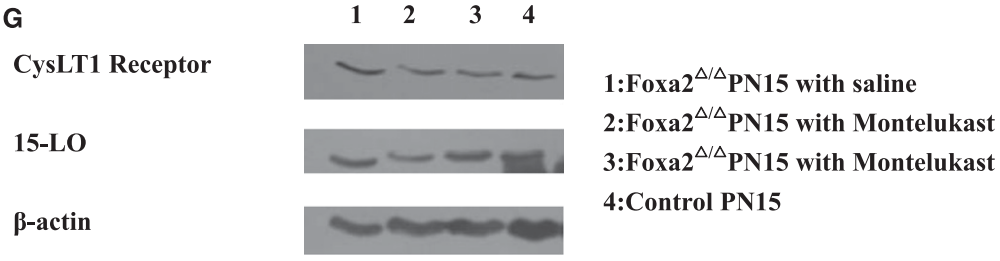


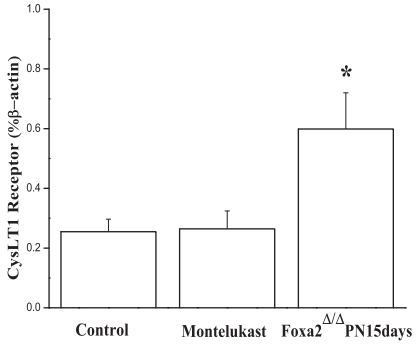












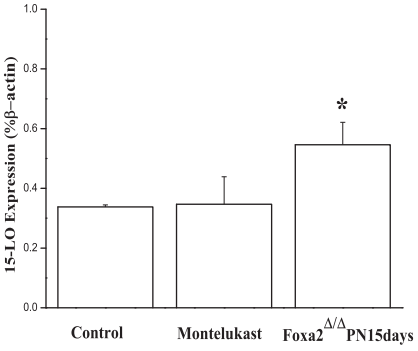


Figure 6. (*Continued*).

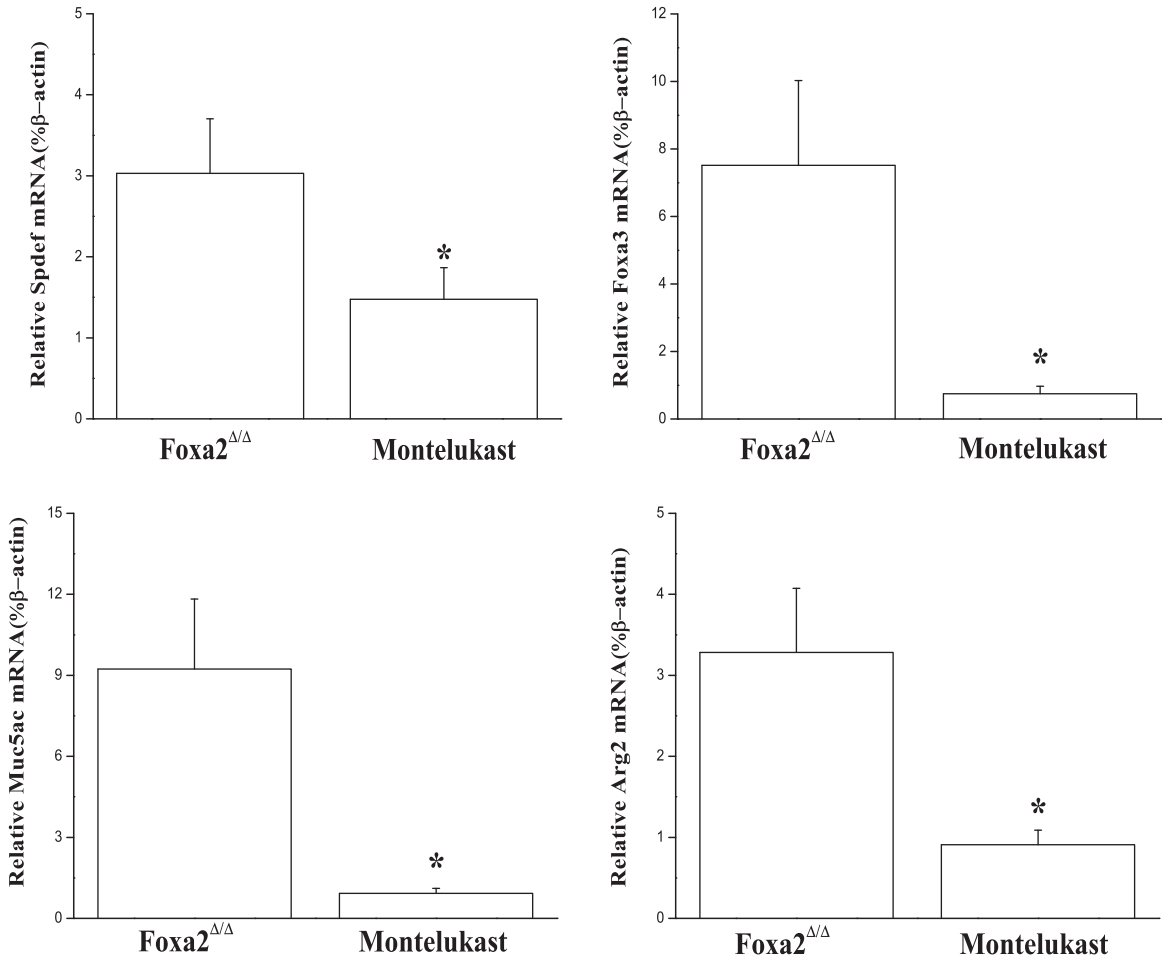


Figure 7. Montelukast inhibited mucus metaplasia induced by Foxa2 deletion. Montelukast treatment from PN5–PN15 significantly inhibited *Spdef*, *Foxa3*, *Muc5ac*, and *Agr2* mRNAs in lungs of Foxa2-deficient mice. Data represent means ± SEMs ($n = 5$ for each genotype) according to quantitative RT-PCR. *$P < 0.05$, versus control values determined by the Student *t* test (two-tailed).

inhibited by Foxa2 *in vitro*. Together, these data indicate that Foxa2 is required in the airway epithelium during lung development to regulate the lipoxygenase pathway. Activation of the lipoxygenase pathway may induce Th2-mediated pulmonary inflammation via an increased production of LTs, and other lipoxygenase products that recruit inflammatory cells into the lungs enhance Th2 cytokine expression and mucus production (23, 24). The finding that inhibition of the $CysLT_1$ receptor with montelukast inhibited Th2 inflammation after the deletion of Foxa2 demonstrates the important role of the LT pathway in this Th2-mediated inflammation. Together, these data indicate that both the respiratory epithelium and the lipoxygenase signaling pathway influence Th2-mediated inflammation during postnatal development of the lung.

CysLTs have a clearly defined role in asthma, perpetuating airway inflammation, smooth muscle constriction, and mucus production, and causing airflow obstruction. CysLTs also increase vascular permeability, and contribute to airway remodeling in murine asthma models (23). LTs promote allergic airway inflammation through regulating the expression of Th2 cytokines in animal models (25). Furthermore, LTE4 and LTD4 stimulate the production of Th2 cytokines in human Th2 cells (26). In the *Ltc4 s*^{null} mice, the production of IL-4, IL-5, and IL-13 after allergen stimulation was reduced (12), indicating that CysLTs play a role in inducing Th2 cytokine expression. Th2 cytokines regulate $CysLT_1$ receptor expression and signaling (26). In human lung macrophages, IL-13 increased CysLT synthesis (11), and IL-13 also enhanced the expression of the principal receptor for CysLTs, $CysLT_1$ (26). In our study, the loss of *Foxa2* in lung epithelial cells increased both Th2 cytokine and CysLT production. LTs promote the production of Th2 cytokines, and Th2 cytokines may activate the LT pathway. The finding that increased LTs preceded Th2 cytokine expression supports the concept that the suppression of LTs by Foxa2 is a critical determinant of pulmonary Th2 polarization in this model.

Montelukast decreased the expression of IL-4, IL-5, eotaxin-2, and RANTES, but did not alter IL-13 expression. The decreased expression of Th2 cytokines and chemokines after montelukast treatment is likely to result from inhibition of the LT pathway. Surprisingly, IL-13 in BALF was increased during montelukast treatment. A possible mechanism underlying these findings may be related to the effect of montelukast inhibition of the LT pathway in inflammatory cells expressing IL-5, IL-4, eotaxin-2, and RANTES. The continued expression of IL-13 by subsets of cells lacking the $CysLT_1$ receptor may explain the continued expression of IL-13. IL-13 exerts pleiotropic effects in the lung, including its central role in the development of airway hyperresponsiveness and tissue remodeling (27). IL-13 increased in lungs of patients with asthma (24). IL-13 promotes Th2 inflammatory cell recruitment and airway hypersecretion characteristic of asthma pathology (28, 29). IL-13 mediates its effects by binding to the IL-13 receptor, which is a multimeric complex made up of IL-

4Rα and IL-13Rα1 (30). Eosinophilic inflammation in *Foxa2*$^{\Delta/\Delta}$ mice was significantly inhibited by the anti–IL-4Rα antibody (3), indicating that the IL-4Rα pathway plays a key role in this Th2-mediated pulmonary inflammation. Our present findings suggest that Th2 cytokine activation occurs after the up-regulation of the LT pathway, supporting the concept that IL-4Rα activation may take place downstream from the LTs in this animal model. The inhibition of LTs inhibits IL-13–mediated bronchopulmonary hyperreactivity, inflammation, and mucosal metaplasia (24, 31), supporting the concept that many effects of IL-13 are mediated through the LT pathway in the setting of allergic asthma.

The loss of Foxa2 in respiratory epithelium enhanced myeloid dendritic-cell (DC) recruitment and activation (3). However, the mechanisms underlying this recruitment remain unknown. Aeroallergen exposure is routinely used to induce Th2 pulmonary inflammation in mice. Splenic cells were activated by antigen stimulation (32). In the present study, splenic lymphocytes were not activated in *Foxa2*$^{\Delta/\Delta}$ mice, indicating that the inflammation was limited to the lungs. Because LTs regulate DC activation, migration, and function (33), the activation of DCs in *Foxa2*$^{\Delta/\Delta}$ mice may result from spontaneous activation of the LT pathway in the absence of Foxa2.

Among the proinflammatory lipid mediators derived from arachidonic acid, LTs are considered to be among the most important inflammatory mediators involved in the pathogenesis of asthma (23). In the developing lung, we demonstrated that the conditional deletion of Foxa2 in respiratory epithelial cells up-regulated the LT pathway and other lipoxygenases, supporting a direct role for Foxa2 in regulating lipid metabolism during postnatal development of the lung. Alox15 promoter activity was directly inhibited by Foxa2. Because Alox15 was found to promote Th2-mediated pulmonary inflammation (15), increased Alox15 expression may promote Th2-mediated pulmonary inflammation after Foxa2 deletion in airway epithelium. Although Alox5 expression increased after Foxa2 deletion in airway epithelial cells, Foxa2 increased the transcription of Alox5 *in vitro*. This result indicates that the increased expression of Alox5 after Foxa2 deletion may not be regulated directly by Foxa2. Alternatively, the regulation of Alox5 gene expression is dependent on cell type and regulatory elements not present in the *Alox5* gene promoter used in the present study.

Antagonists for the $CysLT_1$ receptor have been developed for the treatment of Th2 inflammation diseases, including asthma. Montelukast was recommended for the treatment of mild to severe asthma by the Global Initiative for Asthma (34, 35). In previous studies, Foxa2 expression was decreased in lung tissue from patients with asthma (36). Foxa2 suppressed goblet-cell metaplasia in allergen-sensitized mice (3), and in the present study, the spontaneous activation of the LT pathway was found in Th2-mediated pulmonary inflammation caused by the deletion of *Foxa2* in the airway epithelium during lung development. Lung inflammation and goblet-cell metaplasia were inhibited by montelukast. We conclude that the LT pathway plays an important role in Th2-mediated pulmonary inflammation during lung development.

Author disclosures are available with the text of this article at www.atsjournals.org.

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Foxa2 Regulates Leukotrienes to Inhibit Th2-mediated Pulmonary Inflammation

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Yangyan He, Jeffrey A. Whitsett, Fengming Luo

Online Data Supplement

Supplemental Methods

Western blot analysis

Total protein was extracted with Radio-Immunoprecipitation Assay (RIPA) lysis buffer. Protein concentration of the supernatant was determined by bicinchoninic acid (BCA) method. 25ug protein separated by SDS-PAGE, transferred, and immobilized on nitrocellulose membranes. Membranes were incubated with goat anti-mouse Alox15 (1:100, Santa Cruz, CA, USA) or $CysLT_1$ receptor (1:200, Santa Cruz) antibody. Blots were incubated for 1 h at room temperature with a horseradish peroxidase-conjugated rabbit anti-goat IgG (Chemicon, Temecula, CA, USA) secondary antibody. Signals were visualized using enhanced chemiluminescence (ECL) plus chemiluminescence substrate (Amersham, Piscataway, NJ, USA). Western blotting density was determined with the Bio-Rad Quantity One 1-D analysis software (Bio-Rad, Hercules, CA, USA).

Plasmid construction and luciferase reporter assay

The Alox15 and Alox5 promoter-luciferase constructs were made with pGL3 plasmids. The promoter of mouse *Alox15* genes (from-34 to -1697, ENSMUST00000019068) was amplified from mouse genomic DNA with PCR (forward primer: ACC CAA GAG GAG TCC AGG AT, reverse primer: AGA GGG GTA GAC GGG CTA AA). The products of PCR were cloned into pGL3 with restriction enzyme XhoI and SacI.

The promoter of mouse *Alox5* genes (from-228 to -1815, ENSMUSG00000025701) was amplified from mouse genomic DNA with PCR (forward primer: GAA GCC AGG TCT GCT TTG TC, reverse primer: TCC TTC TCT CCT GTG GCA CT). The products of PCR were cloned into pGL3 with restriction enzyme Xhol and KpnI. The constructs were transiently transfected into H441 cells with Lipofectamine 2000 (Life Technologies, Grand Island, NY), according to the manufacturer's protocols. Forty-eight hours after the transfection, luciferase activity was assessed and normalized for co-transfection efficiency by β-galactosidase activity. All transfections were performed in triplicate.

RNA extraction and quantitative real-time RT-PCR

Reverse transcriptase (RT) was performed with Rever Tra Ace qPCR RT Kit (TOYOBO, Shangthai, China). Quantitative real-time PCR (qPCR) analysis was performed with a Bio-Rad CFX real-time sequence detection system according to manufacturer's instructions. Beta-actin was used as control. The $2^{-\Delta\Delta}$CT method was used to analyze data. The primers for qPCR are listed in supplementary data (Table E1).

Splenocyte activation and cytokines assay

Spleens were gently crushed and single cell suspensions were made by passing the spleen through a 40 μm nylon filter. Following centrifugation, the red blood cells were lysed using osmotic lysis. Splenocytes were then cultured in RPMI 1640 growth medium (GIBCO BRL, Gaithersburg, MD, USA) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 5×10^{-5} M 2-mercaptoethanol, and 5% feotal bovine serum. Cell cultures were maintained at 37°C in a humidified incubator containing 95% air and 5% CO2. Anti-CD3 and anti-CD28 (Pharmingen, San Diego, CA, 5μg/ml) were then added to the cultured cells and cell supernatants were collected 48 hrs following activation. Supernatants were stored at -80°C until assessed for cytokine content and used for IL-13, IL-4 and IFN-γ measurements using a commercially available ELISA kit according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

Supplemental Figure Legends

Fig. E1 Deletion of *Foxa2* in respiratory epithelium did not change Th1 cytokines expression in the lung PN15. TNF-α and IL-1β mRNA expression(A) and protein level (B)were not significantly different between control and Foxa2$^{\Delta/\Delta}$ mice at PN15 measured by qPCR. And ELISA (n=5 of each genotype).

Fig. E2 COX-2 pathway productions in the lung after Foxa2 deletion in respiratory epithelium. COX-2 pathway productions, PGH2 and PGE2, were not

different between control and Foxa2$^{\Delta/\Delta}$ mice at PN15 measured by ELISA (n=5 of each genotype).

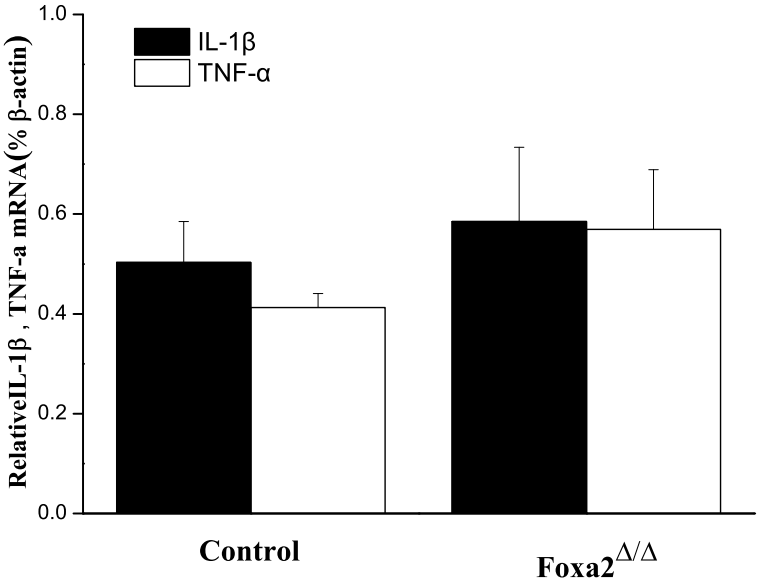
Supplemental Table

Table E1 primers for qPCR

Primer sequence5'→3'			
gene	sense	Anti-sequence	Product length(bp)
IL-4	5'-AGACTCTTTCGGGCTTTTCG-3'	5'-TGATGCTCTTTAGGCTTTCC-3'	106bp
IL-13	5’-GAGGAGGGTTGAGGAGGAAG-3’	5’-TTTCTGTAGGGATGGGATGG-3’	136bp
IL-5	5’-GTGTGAATGAGAGCCAGCCA-3’	5’-TGCTTTCTGTTGGCATGGGG-3’	106bp
TNF-α	5’-AGGAGGGAGAACAGAAACTC-3’	5’-GTGAGTGAAAGGGACAGAAC-3’	90bp
IL-1β	5’-GTACATCAGCACCTCACAAG-3’	5’CAGTCCAGCCCATACTTTAG-3’	116bp
ALOX5	5’-CACGGGGACTACATCGAGTT-3’	5’-TACGTCTGTGCTGCTTGAGG-3’	127bp
ALOX15	5’-AGTTCCTCCTCCCTGTGGTT-3’	5’-CAGGGATCGGAGTACACGTT-3’	134bp
FLAP	5’-CGTAGATGCGTACCCCACTT-3’	5’-CTCCCAGATAGCCGACAAAG-3’	131bp
Muc5ac	5’-ACAGCATCTCCATGTACTCC-3’	5’-GTAGCTGAAGGTCTGACTGG-3	131bp
LTC4s	5’-TCTTCCGAGCCCAGGTAAAC-3’	5’-ACAGGTAGAACAGTCCGCAC-3’	117bp
Foxa3	5’-CCATTGGCTACTGTGGTAAC--3’	5’-GGCAGCTTTCATAGTACACC-3’	148bp
Arg2	5’-GTCAGCTCTGGATCTTGTTGA-3’	5’-GTCTGACCAAAACTTGAAGCA-3’	111bp
spdef	5’-CTGCTACTGTTCCCAGATGT-3’	5’-GAGAGGTAGAAAGCAGACAGG-3’	137bp
Foxa2	5'-GTGGCCTAAGCGAGCTAAAG-3'	5'-TAATGGTGCTCGGGCTTCAG-3'	160bp
Actin	5'-ACTATCGGCAATGAGCGGTTC-3'	5' ATGCCACAGGATTCCATACCC-3'	77bp

Fig E1

A


IL-1β
TNF-α
RelativeIL-1β , TNF-a mRNA(% β-actin)
1.0
0.8
0.6
0.4
0.2
0.0
Control
Foxa2Δ/Δ

B

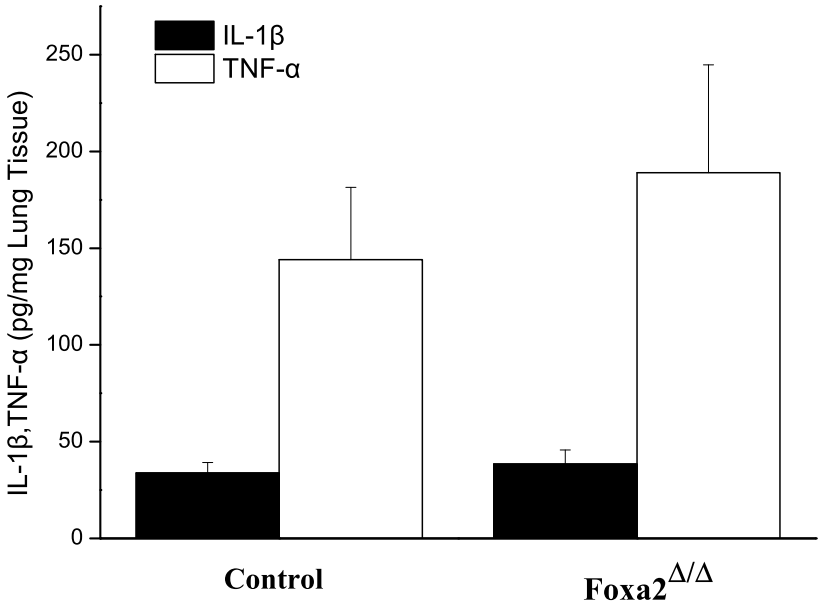
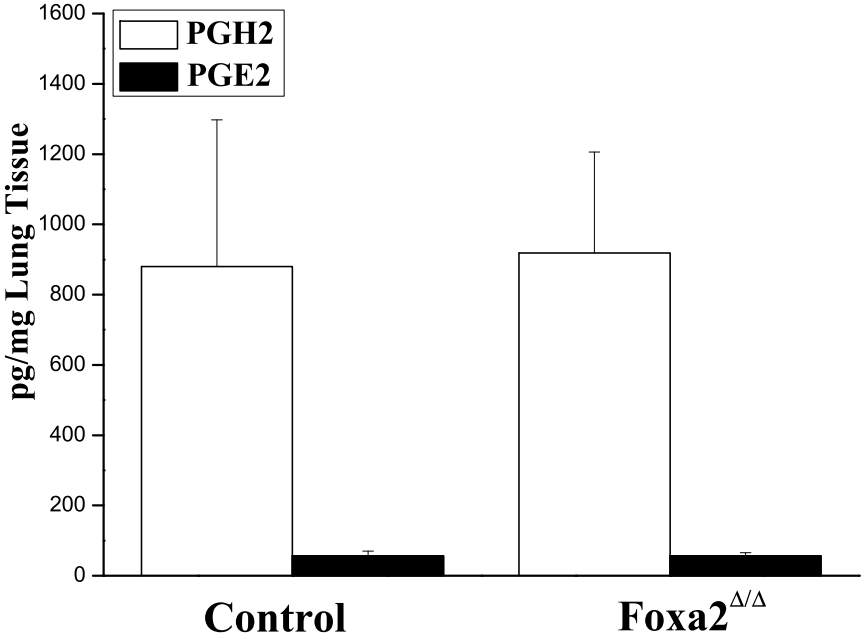

IL-1β
TNF-α
IL-1β,TNF-α (pg/mg Lung Tissue)
250
200
150
100
50
0
Control
Foxa2Δ/Δ

Fig E2


PGH2
PGE2
pg/mg Lung Tissue
1600
1400
1200
1000
800
600
400
200
0
Control
Foxa2Δ/Δ