

Importance of group X-secreted phospholipase A₂ in allergen-induced airway inflammation and remodeling in a mouse asthma model

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Arachidonic acid metabolites, the eicosanoids, are key mediators of allergen-induced airway inflammation and remodeling in asthma. The availability of free arachidonate in cells for subsequent eicosanoid biosynthesis is controlled by phospholipase A₂s (PLA₂s), most notably cytosolic PLA₂-α. 10 secreted PLA₂s (sPLA₂s) have also been identified, but their function in eicosanoid generation is poorly understood. We investigated the role of group X sPLA₂ (sPLA₂-X), the sPLA₂ with the highest in vitro cellular phospholipolysis activity, in acute and chronic mouse asthma models in vivo. The lungs of sPLA₂-X^{-/-} mice, compared with those of sPLA₂-X^{+/+} littermates, had significant reduction in ovalbumin-induced infiltration by CD4⁺ and CD8⁺ T cells and eosinophils, goblet cell metaplasia, smooth muscle cell layer thickening, subepithelial fibrosis, and levels of T helper type 2 cell cytokines and eicosanoids. These data direct attention to sPLA₂-X as a novel therapeutic target for asthma.

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Abbreviations used: BAL, bronchoalveolar lavage; cysLT, cysteinyl leukotriene; cPLA₂-α, cytosolic group IVA PLA₂; EIA, enzyme immunoassay; i.n., intranasal; LTB₄, LTC₄, LTD₄, and LTE₄, leukotriene B₄, C₄, D₄, and E₄, respectively; MOX, methoxime; PAS, periodic acid Schiff; PGD₂ and PGE₂, prostaglandin D₂ and E₂, respectively; PLA₂, phospholipase A₂; R_L, lung resistance; RT, real time; sPLA₂, secreted PLA₂; sPLA₂-X, group X sPLA₂.

Allergen-induced airway inflammation in asthma is characterized by eosinophil and CD4⁺ and CD8⁺ T cell infiltration of the airways and accompanied by airway structural changes (i.e., airway remodeling), including goblet cell metaplasia, increased smooth muscle mass, and subepithelial fibrosis from excessive deposition of extracellular matrix components such as collagen and laminin (for review see references 1, 2). Leukotrienes and prostaglandins are, respectively, 5-lipoxygenase and cyclooxygenase metabolites of arachidonic acid (i.e., eicosanoids) that are important in the pathogenesis of asthma. Release of 5-lipoxygenase products leukotriene B₄ (LTB₄), the cysteinyl leukotrienes (cysLTs) C₄, D₄, and E₄, and cyclooxygenase products prostaglandin D₂ (PGD₂) and thromboxane A₂ from inflamed airways leads to airway hyperresponsiveness and airflow obstruction with mucus glycoproteins. Evidence from animal models

and patients with asthma suggest that these eicosanoids are key molecules that promote airway inflammation as potent chemoattractants for eosinophils, T cells, and other inflammatory cells; cause plasma extravasation and edema; modulate airway smooth muscle cell function; and induce release of extracellular matrix components (3–5).

The availability of free arachidonate in cells and, thus, the biosynthesis of leukotrienes and other eicosanoids, including prostaglandins, is tightly controlled by the regulated action of phospholipase A₂s (PLA₂s) that release this fatty acid by hydrolysis of the *sn*-2 ester of glycerophospholipids present as major components of cell membranes. Mammalian cells contain multiple types of PLA₂s (6), but it is generally accepted that cytosolic group IVA PLA₂-α (cPLA₂-α; also known as group IVA PLA₂; PLA2G4A) plays a pivotal role in agonist-mediated arachidonate release for the biosynthesis of the eicosanoids. This is based on studies

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with cPLA₂-α inhibitors (7–10) and cPLA₂-α-deficient mice (11–13). The mammalian genome also encodes 10 secreted PLA₂s (sPLA₂s). The role of these enzymes in eicosanoid biosynthesis is much less clear. Disruption of the group V sPLA₂ gene (*PLA2G5*) in mice leads to ~50% reduction in the amount of LTC₄ and PGE₂ production in zymosan-stimulated peritoneal macrophages (14). Group IIA, V, and X sPLA₂s have been shown to coordinate with cPLA₂-α to augment arachidonate release from cultured cells (15–20), but the mechanism for this coordinate action between cPLA₂-α and sPLA₂ is not known.

A systematic investigation of the interfacial kinetic and binding properties of the full set of mouse and human sPLA₂s shows that the group X sPLA₂ (sPLA₂-X; *PLA2G10*) stands out as having the highest phospholipolysis activity when added to cultured cells (21, 22). Based on these findings and the fact that sPLA₂-X is expressed in human (23, 24) and mouse lungs (this study), we investigated the possible role of sPLA₂-X in a mouse asthma model by generating a sPLA₂-X-deficient mouse for studies of allergen-induced airway inflammation and remodeling. In this initial study, we explored an animal disease model rather than experiments with single cell types in vitro, because sPLA₂-X is a secreted enzyme and can potentially act extracellularly on many, if not all, mammalian cell types to liberate arachidonic acid. Therefore, studies with cultured mammalian cells cannot shed sufficient light on the behavior of sPLA₂-X in tissues. This is a different situation than with cPLA₂-α that acts in the cytoplasm.

RESULTS

Generation and characterization of the sPLA₂-X^{-/-} mouse

Mutation of the mouse sPLA₂-X gene was accomplished by retroviral-mediated insertion of an exon-trapping cassette (see Materials and methods). DNA sequence analysis revealed that the trapping cassette is located in the intron between exons 3 and 4 of the sPLA₂-X gene. Real-time (RT)-PCR analysis using primers hybridizing to exons 2 and 4 and cDNA made from mouse testis, colon, and stomach revealed ~1,000-fold reduction in sPLA₂-X mRNA levels in the sPLA₂-X^{-/-} mouse versus the wild-type littermate. We also looked for sPLA₂-X protein in stomach extracts using a sensitive time-resolved fluorescence immunoassay (25). When 24 μg of stomach lysate protein was analyzed from a sPLA₂-X^{+/+} mouse, 0.2 ng sPLA₂-X protein was detected, whereas no sPLA₂-X protein was detected when 34 μg of lysate protein from a sPLA₂-X^{-/-} mouse was used.

Allergen-induced airway inflammation and expression of sPLA₂-X

Important insights into the mechanisms of chronic inflammation and remodeling in asthma have come from animal models that reproduce key morphologic and physiologic features of human asthma. To study the in vivo effects of sPLA₂ deficiency on the asthma phenotype, we used OVA as a model allergen to induce allergen-specific pulmonary disease in

wild-type and sPLA₂-X^{-/-} mice. In the acute asthma protocol (3), OVA-sensitized mice challenged with four doses of OVA display, by day 29, a disease strikingly similar to allergen-induced human asthma, including (a) circulating levels of allergen-specific IgE; (b) a marked influx of eosinophils and T cells into the airways; (c) increased levels of mucus glycoproteins, Th2 cell cytokines (IL-4, IL-5, and IL-13), and leukotrienes and other eicosanoids in bronchoalveolar lavage (BAL) fluid; and (d) pulmonary hyperreactivity to methacholine compared with saline-treated control mice. When the intranasal (i.n.) OVA challenges are continued periodically in the chronic asthma protocol (4, 26), by day 76 the mice have striking features of airway remodeling, including (a) goblet cell metaplasia, (b) increased smooth muscle cell layer

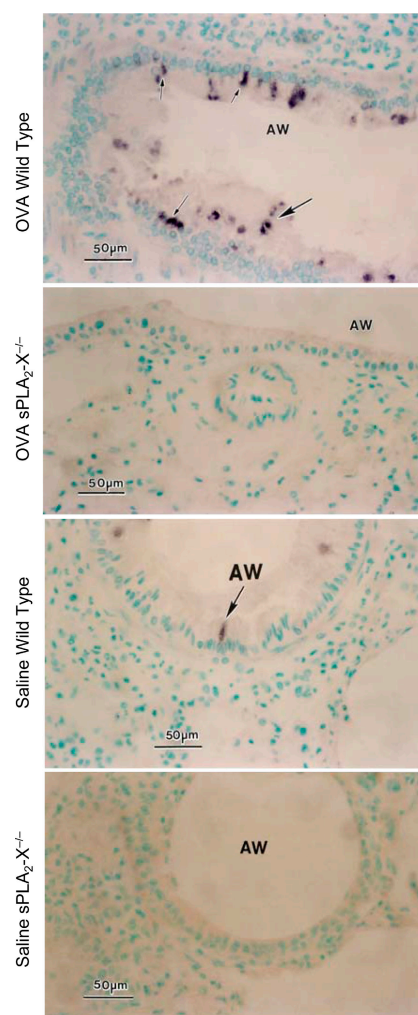


Figure 1. Absent sPLA₂-X expression in lungs of sPLA₂-X^{-/-} mice. Lung tissue was obtained on day 29 (acute asthma model) from sPLA₂-X^{+/+} and sPLA₂-X^{-/-} mice treated with either OVA or saline and examined by immunocytochemistry for sPLA₂-X expression. Arrows indicate peroxidase-positive cells in the airways (AW) expressing sPLA₂-X. Additional airway sections of OVA-treated sPLA₂-X^{+/+} mice are shown in Fig. S1. Absent sPLA₂-X expression in the splenic tissue of sPLA₂-X^{-/-} mice is shown in Fig. S2. Bars, 50 μm.

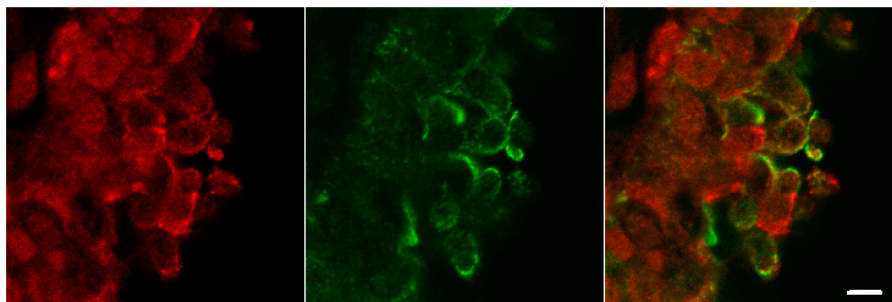


Figure 2. Colocalization of sPLA₂-X with airway epithelial cells expressing mucin 5AC glycoprotein in OVA-treated wild-type mice. Red fluorophore (Alexa Fluor 594) localization of sPLA₂-X (left), green

fluorophore (Alexa Fluor 488) localization of mucin 5AC (middle), and colocalization of sPLA₂-X with mucin 5AC-positive airway epithelial cells (right). Bar, 20 μ m.

mass, and (c) extensive subepithelial collagen deposition and airway wall thickening.

sPLA₂-X expression in the lungs and the spleen of sPLA₂-X^{-/-} and wild-type mice in the acute asthma model was examined by immunocytochemistry. sPLA₂-X was expressed in the airway epithelium in the lungs in saline-treated wild-type mice but not in sPLA₂-X^{-/-} mice (Fig. 1). After OVA sensitization and challenge, sPLA₂-X expression greatly increased in the columnar cells lining the airways of wild-type mice but remained undetected in sPLA₂-X^{-/-} mice (Fig. 1 and Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20070029/DC1>). Confocal immunofluorescence microscopy demonstrated colocalization of sPLA₂-X with airway epithelial cells expressing mucin 5AC glycoprotein (Fig. 2). Macrophages contributed to sPLA₂-X expression in the airways, because alveolar macrophages, isolated from BAL fluid cells of OVA-treated wild-type mice (but not sPLA₂-X^{-/-} mice), exhibited strong reactivity for sPLA₂-X by immunocytochemistry (Fig. 3). Splenic mononuclear cells also expressed sPLA₂-X in sPLA₂-X^{+/+} mice but not in sPLA₂-X^{-/-} mice (Fig. S2). No staining reaction for sPLA₂-X was observed when immunocytochemistry was performed with preimmune serum (Fig. S2).

Effect of sPLA₂-X deficiency on allergen-induced recruitment of inflammatory cells into the lungs and hyperresponsiveness

On day 29, 24 h after the final i.n. OVA challenge in animals from each experimental group (i.e., acute asthma model), the effect of sPLA₂-X deficiency on allergen-induced airway inflammation and hyperreactivity was determined. OVA-treated sPLA₂-X^{+/+} mice had a sixfold increase in total cells recovered in BAL fluid compared with the saline group control (Fig. 4 a); ~50% of the BAL fluid cells were eosinophils in the OVA-treated sPLA₂-X^{+/+} mice. The total number of inflammatory cells and eosinophils in the BAL fluid of OVA-treated sPLA₂-X^{-/-} mice was significantly reduced compared with wild-type controls (Fig. 4 a). By light microscopy and morphometry, an influx of eosinophils and mononuclear cells into the lungs around the airways and blood vessels, airway goblet cell metaplasia/mucus hypersecretion, and inter-

stitial edema were observed in OVA-treated sPLA₂-X^{+/+} mice compared with saline controls (Fig. 4, b and c; and Fig. S3, available at <http://www.jem.org/cgi/content/full/jem.20070029/DC1>). This increased infiltration of total inflammatory cells and eosinophils, goblet cell metaplasia/mucus hypersecretion, and edema of the airway interstitium were markedly decreased in OVA-treated sPLA₂-X^{-/-} mice compared with sPLA₂-X^{+/+} mice (Fig. 4, b and c; and Fig. S3). Using invasive plethysmography to determine lung resistance (R_L), airway hyperreactivity to aerosolized methacholine was significantly increased in the OVA-treated wild-type mice compared with saline controls (Fig. 4 d). In contrast, pulmonary responses in the sPLA₂-X^{-/-} mice were not significantly different between the OVA and saline treatment groups (Fig. 4 d). In vivo airway responsiveness to aerosolized methacholine was also determined on day 29 in conscious, freely moving, spontaneously breathing mice using noninvasive whole-body plethysmography, and the same trends for Penh

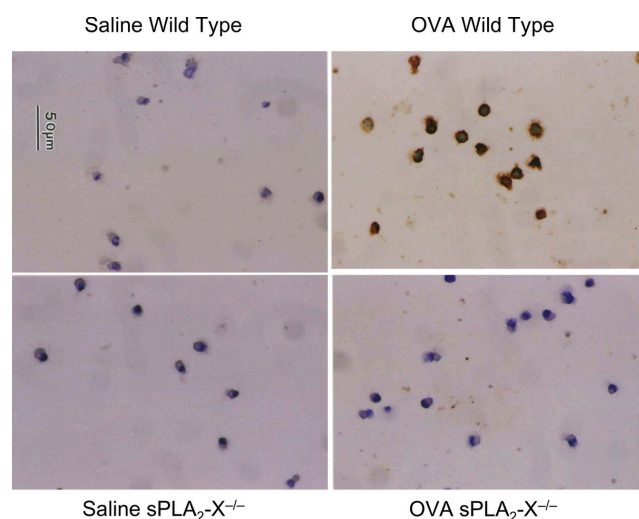


Figure 3. sPLA₂-X expression in alveolar macrophages of wild-type mice. Alveolar macrophages were isolated on day 29 from sPLA₂-X^{+/+} and sPLA₂-X^{-/-} mice treated with either OVA or saline and examined by immunocytochemistry for sPLA₂-X expression. Bar, 50 μ m.

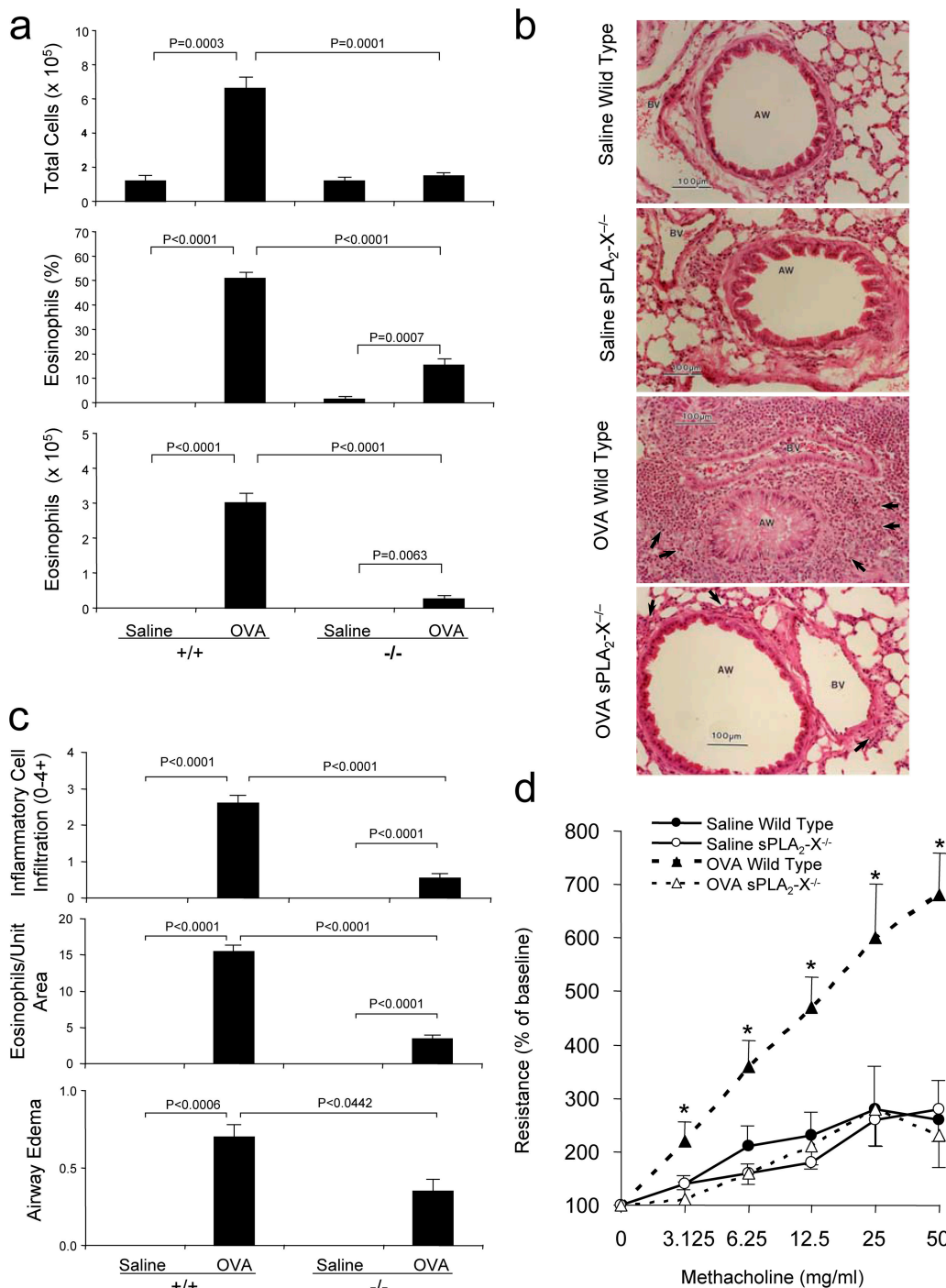


Figure 4. Impaired allergen-induced airway inflammation and hyperreactivity in sPLA₂-X^{-/-} mice. (a) BAL fluid was obtained on day 29 from saline-treated ($n = 5$) and OVA-treated ($n = 9$) sPLA₂-X^{+/+} mice ($+/+$) and saline-treated ($n = 7$) and OVA-treated ($n = 8$) sPLA₂-X^{-/-} mice ($-/-$), and the number of total cells and percentage and number of eosinophils were determined. (b) Lung sections of saline- and OVA-treated sPLA₂-X^{+/+} and sPLA₂-X^{-/-} mice were stained with hematoxylin and eosin. Arrows indicate inflammatory cells. AW, airways; BV, blood vessels. Bars, 100 μ m. (c) The intensity of the total inflammatory cell infiltrate

(0-4+ scale), the number of eosinophils per unit area (2,200 μ m²), and airway edema (0-4+ scale) in the lungs on day 29 were determined by morphometric analysis. (d) Allergen-induced airway hyperresponsiveness was assessed by invasive plethysmography on day 29 in wild-type ($+/+$) and sPLA₂-X^{-/-} ($-/-$) mice as the degree of bronchoconstriction to aerosolized methacholine (0, 3.125, 6.25, 12.5, 25, and 50 mg/ml). R_L was calculated as described in Materials and methods and is shown as the percentage of baseline response to aerosolized normal saline. *, $P < 0.05$ versus saline. Data in a, c, and d represent the mean $\pm$ SEM.

values were observed as for the invasive R_L measurements (unpublished data).

Effect of sPLA₂-X deficiency on allergen-induced airway remodeling

Wild-type mice challenged with OVA periodically over a 75-d period (i.e., chronic asthma model) had persistent airway infiltration by eosinophils and edema (Fig. 5 a; and Fig. S4 and Fig. S5, available at <http://www.jem.org/cgi/content/full/jem.20070029/DC1>), goblet cell metaplasia and mucus hypersecretion on day 76 (Fig. 5, a and b), and increased airway smooth muscle mass and collagen deposition (Fig. 6, a and b) compared with saline controls. These features of allergen-induced airway remodeling were significantly reduced in sPLA₂-X^{-/-} mice (Fig. 5, a and b; and Fig. 6, a and b).

Effect of sPLA₂-X deficiency on Th2 responses

A molecular hallmark of asthma is Th2 cytokine expression. We examined the effect of sPLA₂-X deficiency on Th2 cell-driven OVA-specific IgE levels, trafficking of T cells to the lungs, and pulmonary Th2 versus Th1 cytokine expression in both the acute and chronic mouse asthma models. Circulating levels of allergen-specific IgE in blood were reduced on day 29 in sPLA₂-X^{-/-} mice compared with wild-type controls after OVA treatment (Fig. 7 a). A substantial in-

crease in the total number of T cells and also in the numbers of both CD4⁺ and CD8⁺ cells was seen in the BAL fluid of wild-type mice after OVA treatment on day 29 (Fig. 7 b). In contrast, negligible trafficking of T cells to the BAL fluid was observed in allergen-challenged sPLA₂-X^{-/-} mice (Fig. 7 b). By RT-PCR, gene expression of the Th2 cytokines IL-4, IL-5, and IL-13 was markedly increased in whole-lung tissue of OVA-sensitized/challenged sPLA₂-X^{+/+} mice compared with saline-treated controls on day 76 (Fig. 7 c). In OVA-treated sPLA₂-X^{-/-} mice compared with wild-type controls, gene expression of IL-4, IL-5, and IL-13 was significantly reduced (Fig. 7 c). No significant change in gene expression of the Th1 cytokines IL-2 and IFN- γ , the lymphocyte-activating factor IL-1 β , or the Th1-inducing cytokine IL-12 was seen in either wild-type or sPLA₂-X^{-/-} mice after OVA treatment compared with saline controls (Fig. 7 c). Significant levels of IL-4, IL-5, and IL-13 proteins were found in the BAL fluid of OVA-treated sPLA₂-X^{+/+} mice compared with the saline control group on day 76 (Fig. 7 d). In the BAL fluid of OVA-treated sPLA₂-X^{-/-} mice, IL-4 was not detected, and IL-5 and IL-13 levels were significantly reduced compared with wild-type controls (Fig. 7 d). IL-1 β , IL-2, IL-12 (p70), and IFN- γ were not detected in the BAL fluid of wild-type and sPLA₂-X^{-/-} mice after either saline or OVA treatment. Similar reductions in the pulmonary Th2 cytokine responses were observed in sPLA₂-X^{-/-} compared

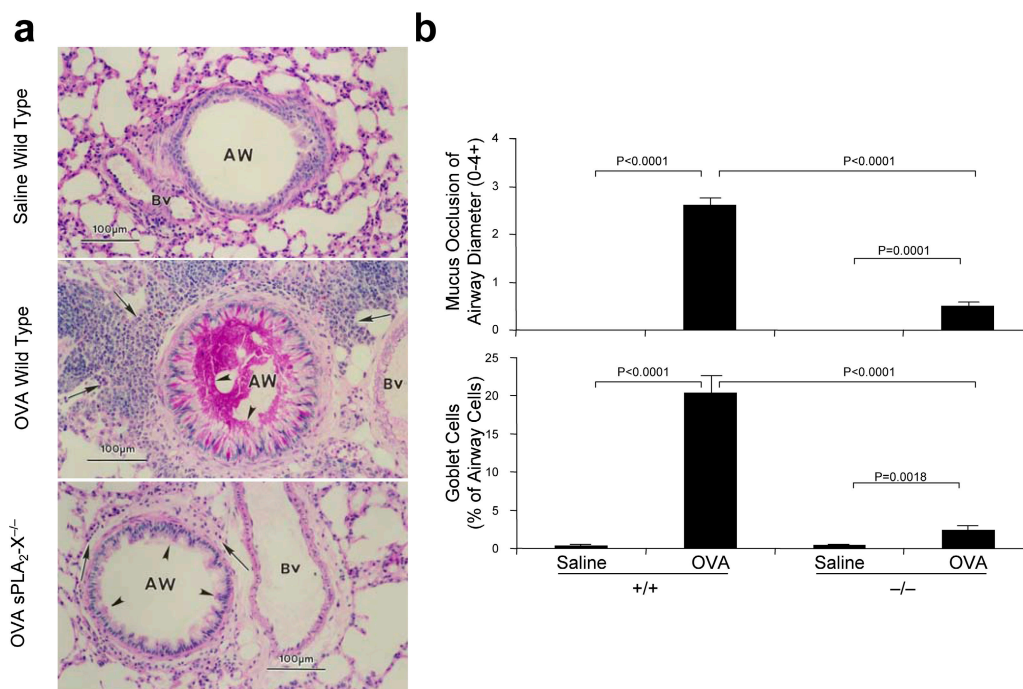


Figure 5. sPLA₂-X deficiency decreases allergen-induced goblet cell metaplasia. (a) Lung tissue was obtained on day 76 (chronic asthma model) from sPLA₂-X^{+/+} and sPLA₂-X^{-/-} mice treated with either saline or OVA and stained with alcian blue/PAS. Arrowheads indicate goblet cells, and arrows indicate inflammatory cells. AW, airways; BV, blood vessels. Bars, 100 μ m. (b) The occlusion of airway diameter by mucus (0-4+ scale)

and the percentage of total airway epithelial cells positive for mucus glycoproteins by alcian blue/PAS staining in the lungs were determined by morphometric analysis ($n = 4$ for each group). Impaired allergen-induced airway goblet cell metaplasia and mucus hypersecretion in sPLA₂-X^{-/-} mice on day 29 in an acute asthma model are shown in Fig. S3. Data represent the mean $\pm$ SEM.

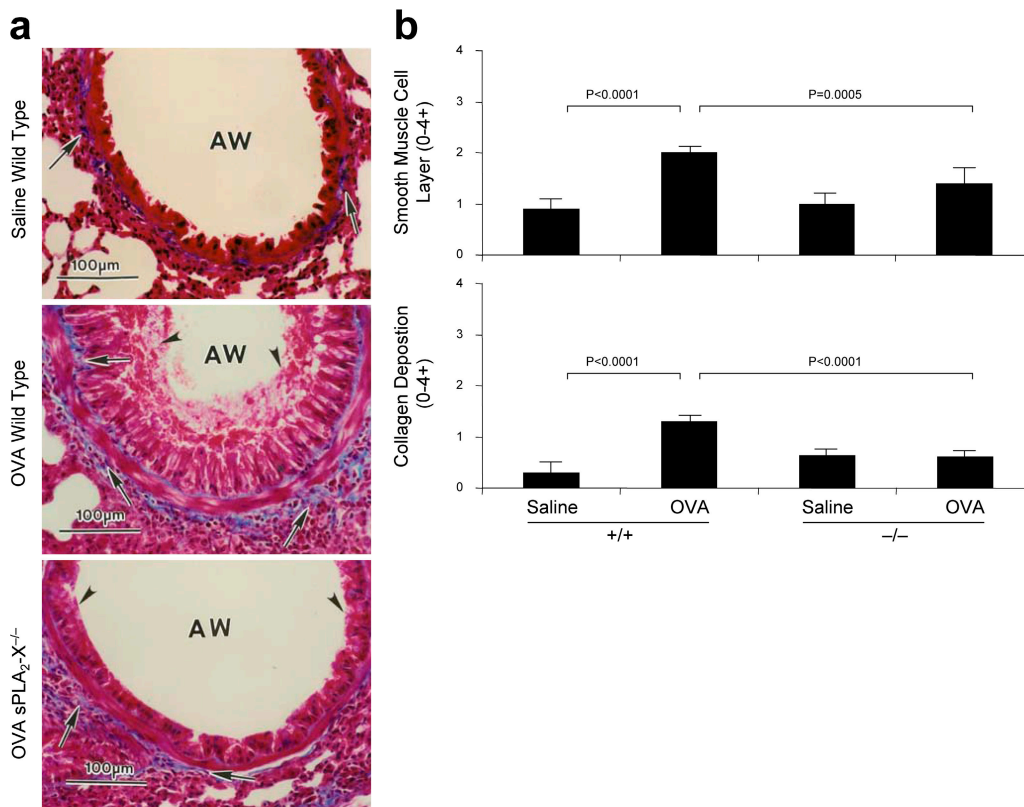


Figure 6. sPLA₂-X deficiency reduces allergen-induced increased smooth muscle cell layer mass and subepithelial fibrosis. (a) Lung tissue was obtained on day 76 from sPLA₂-X^{+/+} and sPLA₂-X^{-/-} mice treated with either saline or OVA and stained with Masson's trichrome stain. Arrows indicate collagen and arrowheads indicate mucus. AW, airways.

Bars, 100 μm. (b) Airway smooth muscle cell layer mass and subepithelial collagen deposition were determined by morphometry (0-4+ scale). Impaired allergen-induced BAL fluid eosinophilia and airway tissue inflammation on day 76 in sPLA₂-X^{-/-} mice in a chronic asthma model are shown in Fig. S4 and Fig. S5, respectively. Data represent the mean ± SEM.

with wild-type mice in the acute asthma model (unpublished data).

To examine the effect of sPLA₂-X deficiency on systemic CD4⁺ T cell-mediated production of Th2 cytokines in response to antigenic challenge, we used flow cytometric analysis to examine production of IL-4, IL-5, and IL-10, as well as the proinflammatory cytokines IFN-γ and TNF-α, upon overnight in vitro stimulation of splenic CD4⁺ T cells from OVA-immunized and unimmunized mutant and wild-type mice in the presence of OVA. Comparable antigen-specific production of IL-5 (Fig. 8 a) and IL-10 (Fig. 8 a) was detected in both mutant and wild-type T cells, as indicated by ~2–2.5-fold increase in the percentage of cytokine-producing CD4⁺ T cells in the presence versus absence of OVA. In contrast to IL-5 and IL-10, antigen-specific production of IL-4, IFN-γ, and TNF-α was not detected using intracellular cytokine staining, suggesting that the frequency of OVA-specific CD4⁺ T cells producing these cytokines was below the detection level (unpublished data). Therefore, we used a single-cell immunospot assay (ELISPOT) to measure their OVA-stimulated production of IL-4 and IFN-γ. In agreement with flow cytometric analyses of IL-5 and IL-10

production, we found no difference in their ability to produce IL-4 (Fig. 8 c) and IFN-γ (Fig. 8 d). Further, there was a comparable ability to produce IL-4 by splenic CD4⁺ T cells from sPLA₂-X^{-/-} mice and wild-type controls in response to anti-CD3/anti-CD28 stimulation (Fig. 8 e). Thus, sPLA₂-X deficiency does not result in a general repression of Th2 or Th1 cytokine production.

Effect of sPLA₂-X deficiency on eicosanoid release

To assess the effect of sPLA₂-X deficiency on arachidonic acid release, we used an acute asthma model to measure the levels of eicosanoids that are derived from this fatty acid, specifically via the cyclooxygenase pathway leading to PGD₂ and PGE₂ and via the 5-lipoxygenase pathway leading to LTB₄ and cysLTs C₄, D₄, and E₄ (i.e., total cysLTs). Because of its chemical instability (27), PGD₂ was converted to a stable methoxime (MOX) derivative to prevent its further chemical degradation, and PGD₂-MOX was assayed to determine PGD₂ levels. Because PGE₂ is rapidly and extensively metabolized in vivo (28), both native PGE₂ and its 13,14-dihydro-15-keto metabolite were assayed to determine the total PGE₂ levels produced. In an acute asthma

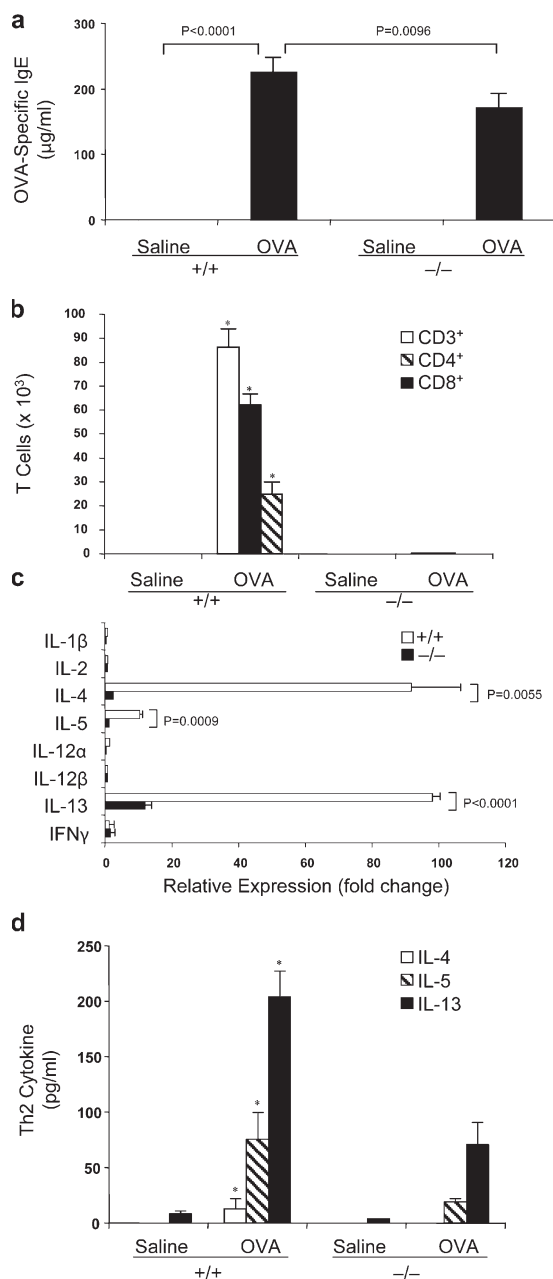


Figure 7. Impaired Th2 cytokine responses in sPLA₂-X^{-/-} mice.

(a) OVA-specific IgE levels were determined in plasma obtained on day 29 from saline- and OVA-treated mGX^{+/+} (+/+) and sPLA₂-X^{-/-} (-/-) mice. (b) The number of CD3⁺, CD4⁺, and CD8⁺ T cells in the BAL fluid on day 29 was determined by flow cytometry. Low cell levels (× 10³) not shown on the graph are as follows: saline-treated wild-type mice (CD3⁺, 0.111 ± 0.001; CD4⁺, 0.083 ± 0.002; and CD8⁺, 0.028 ± 0.002 [reference 1]); saline-treated sPLA₂-X^{-/-} mice (CD3⁺, 0.103 ± 0.002; CD4⁺, 0.079 ± 0.001; and CD8⁺, 0.028 ± 0.003 [reference 2]); and OVA-treated sPLA₂-X^{-/-} mice (CD3⁺, 0.264 ± 0.051; CD4⁺, 0.196 ± 0.025; and CD8⁺, 0.065 ± 0.005 [reference 3]). *, P < 0.05 for both saline versus OVA in +/+ mice and OVA in +/+ mice versus OVA in -/- mice. (c) IL-1β, IL-2, IL-4, IL-5, IL-12α, IL-12β, IL-13, and IFN-γ mRNAs were determined by RT-PCR in whole-lung tissue from sPLA₂-X^{+/+} and sPLA₂-X^{-/-} mice on day 76. (d) IL-4, IL-5, and IL-13 protein levels (pg/ml) were determined in the BAL fluid obtained on day 76 in saline- and OVA-treated sPLA₂-X^{+/+} (+/+) and sPLA₂-X^{-/-} mice (-/-). *, P < 0.05 for both saline versus OVA in +/+ mice and OVA in +/+ mice versus OVA in -/- mice. Data represent the mean ± SEM.

model, PGD₂ (Fig. 9 a), PGE₂ (Fig. 9 b), LTB₄ (Fig. 9 c), and cysLT (Fig. 9 d) levels were each significantly increased in the BAL fluid of sPLA₂-X^{+/+} mice after OVA treatment compared with saline controls with cysLTs >> PGE₂ > LTB₄ > PGD₂. The 13,14-dihydro-15-keto-PGE₂ metabolite represented 91% of total PGE₂ levels in the OVA-treated wild-type mice. As determined by liquid chromatography-electrospray tandem mass spectrometry, the fractional distribution of LTC₄, LTD₄, and LTE₄ was similar in the BAL fluid of OVA- and saline-treated wild-type mice, with LTE₄ the predominant cysLT recovered (i.e., the respective fractional distributions of LTC₄:LTD₄:LTE₄ in OVA- and saline-treated sPLA₂-X^{+/+} mice were 0.11:0.26:0.63 and 0.10:0.25:0.65, respectively). The levels of PGD₂, PGE₂, LTB₄, and cysLTs in the BAL fluid of allergen-challenged sPLA₂-X^{-/-} mice were not significantly different than those of saline-controls (Fig. 9, a-d). The fractional distribution of LTC₄, LTD₄, and LTE₄ in the BAL fluid of OVA- and saline-treated sPLA₂-X^{-/-} mice was the same as that observed for wild-type controls (0.11:0.26:0.63 for OVA-treated sPLA₂-X^{-/-} mice and 0.10:0.25:0.65 for saline-treated sPLA₂-X^{-/-} mice). On day 76 compared with saline controls (chronic model), both cyclooxygenase (i.e., PGE₂) and 5-lipoxygenase (i.e., cysLTs) products of arachidonic acid were substantially increased in the BAL fluid of wild-type but not sPLA₂-X^{-/-} mice after OVA treatment (unpublished data).

DISCUSSION

Our results suggest that sPLA₂-X plays a critical role in the pathogenesis of the asthma phenotype. The major findings of this study are that deficiency of sPLA₂-X in mice substantially reduces allergen-induced (a) airway eosinophil and CD4⁺ and CD8⁺ T cell trafficking, mucus hypersecretion, and hyperresponsiveness to methacholine in an acute model of asthma; (b) airway remodeling (i.e., goblet cell metaplasia, smooth muscle cell layer thickening, and subepithelial fibrosis in the lungs) in a chronic asthma model; and (c) Th2 cytokine and eicosanoid levels in these models of human asthma.

Our data suggest that sPLA₂-X plays a role in eicosanoid generation and that the reduction in eicosanoid generation seen in the sPLA₂-X^{-/-} mice leads to a decrease in Th2 responses. The Th2 cell subset of CD4⁺ T cells has been recognized as pivotal in the development of this allergen-induced airway eosinophilic infiltration, mucus hypersecretion, fibrosis, and hyperreactivity through the secretion of IL-4, IL-5, and IL-13 cytokines. More recently, CD8⁺ T cells have been demonstrated to substantially contribute to these allergen-induced responses by their trafficking to the lungs and local generation of IL-13 (29). We found that sPLA₂-X deficiency

and sPLA₂-X^{-/-} mice (-/-). *, P < 0.05 for both saline versus OVA in +/+ mice and OVA in +/+ mice versus OVA in -/- mice. Data represent the mean ± SEM.

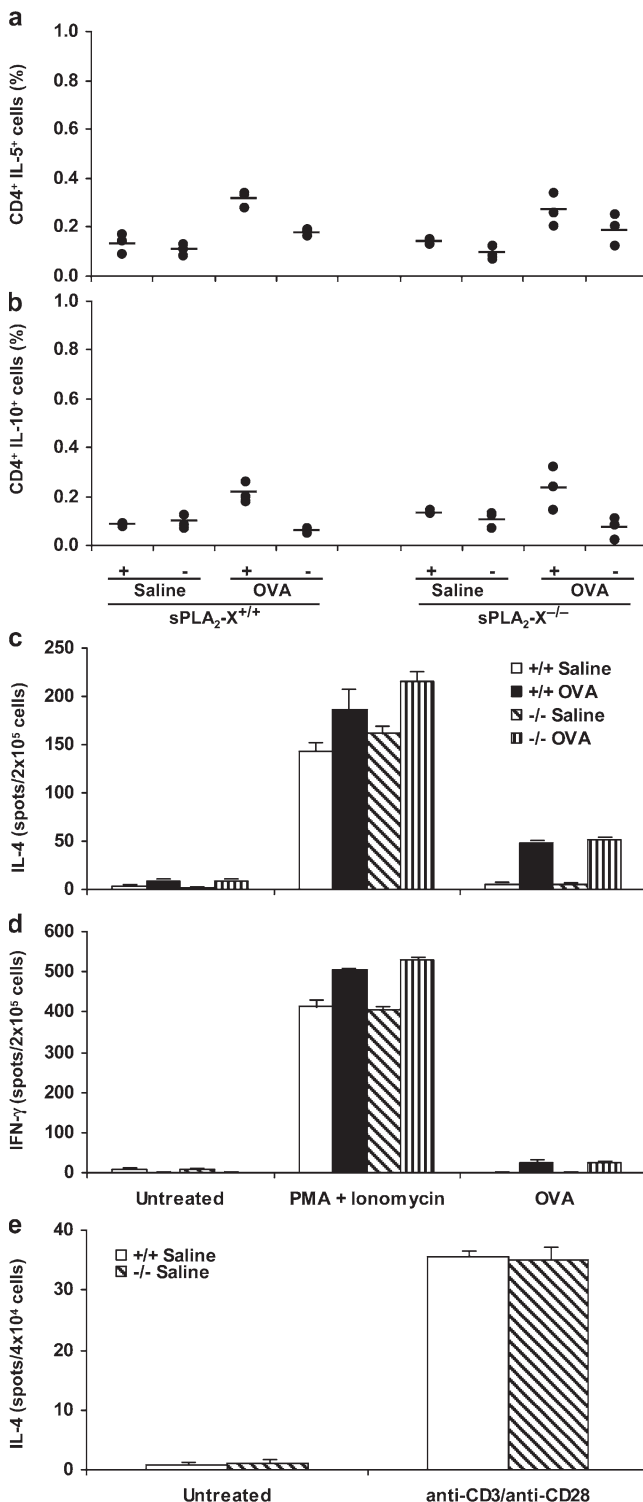


Figure 8. sPLA₂-X deficiency does not affect cytokine production in CD4⁺ T cells. For flow cytometric analysis of intracellular cytokines, splenocytes from saline- and OVA-treated sPLA₂-X^{-/-} and sPLA₂-X^{+/+} mice were cultured with or without 200 μg/ml OVA in the presence of antigen-presenting cells and stained for surface CD4 and the cytokines IL-5 (a) and IL-10 (b). The percentage of cytokine-producing CD4⁺ cells from individual animals is shown. Horizontal bars represent the mean for three different age- and sex-matched animals. + represents cells acti-

resulted in a marked decrease in movement of both CD4⁺ and CD8⁺ T cells to the airway lumen that was associated with diminished pulmonary Th2 cytokine gene and protein expression. Splenic T cells from the sPLA₂-X^{-/-} mice showed normal induction of Th2 and Th1 cytokines with ex vivo stimulation. Thus, the marked impairment in the ability of the mutant mice to recruit allergen-specific T cells to the lungs likely accounts for the observed diminution in pulmonary Th2 cytokine production and inflammation. Among other key effects on the asthma phenotype, IL-13 induces secretion of mucin 5AC glycoprotein, differentiation of ciliated epithelial cells into goblet cells (30), and release of TGFβ, leading to fibrosis (31) in the airways of mice after allergen challenge. IL-4, in the absence of other Th2 cytokines, can also induce typical Th2 responses, including airway goblet cell metaplasia, in a mouse asthma model (32). IL-5 promotes growth and maturation of eosinophil precursors and stimulates the chemotaxis of mature eosinophils, prolonging their survival in allergic inflammatory tissue sites by inhibition of apoptosis (2). In both wild-type and sPLA₂-deficient mice, Th1 cytokines remained low after OVA stimulation.

We found that in sPLA₂-X-deficient mice, there is a reduction in elevated levels of eicosanoids found in OVA-treated wild-type mice, and this likely leads to an amelioration of the asthma phenotype. In mouse models of asthma, specific inhibitors of 5-lipoxygenase, or leukotriene receptor antagonists, considerably reduce indices of allergic airway inflammation, including Th2 cytokine levels (3, 4). The cysLT₁ receptor antagonist montelukast inhibits Th2 cytokine gene and protein expression in the lungs of sensitized mice chronically challenged with OVA (4). Further, established and persistent airway eosinophilia, goblet cell metaplasia, increased airway smooth muscle mass, and subepithelial fibrosis in a mouse model of allergen-induced airway remodeling are reversible by cysLT₁ receptor blockade (26). The cysLTs promote growth of eosinophil progenitors from the blood and bone marrow of atopic individuals (33), stimulate eosinophil chemotaxis, and increase their survival in tissue sites of allergic inflammation. Bronchial smooth muscle cells, with increased cysLT₁ receptor expression after TGFβ and IL-13 treatment, proliferate in response to LTD₄ (34). cysLTs also induce the release of mucus by airway goblet cells (3) and collagen by TGFβ-transformed lung myofibroblasts (35). In IL-13-induced airway inflammation and remodeling in mice, 5-lipoxygenase inhibitors, as well as cysLT₁ and LTB₄

ated in vitro with OVA, and — represents control cells incubated with PBS. For ELISPOT assay of IL-4 (c and e) and IFN-γ (d) production, splenocytes from sPLA₂-X^{+/+} mice treated with saline (+/+) or OVA (+/+) and sPLA₂-X^{-/-} mice treated with saline (-/-) or OVA (-/-) were incubated in the absence (untreated) or presence of either 5 ng/ml PMA/500 ng/ml ionomycin (PMA + ionomycin) or 500 μg/ml OVA (c and d), or CD4⁺ cells isolated from the total splenic cells were incubated in the absence (untreated) or presence of anti-CD3/anti-CD28 antibodies (e). Data represent the mean ± SEM.

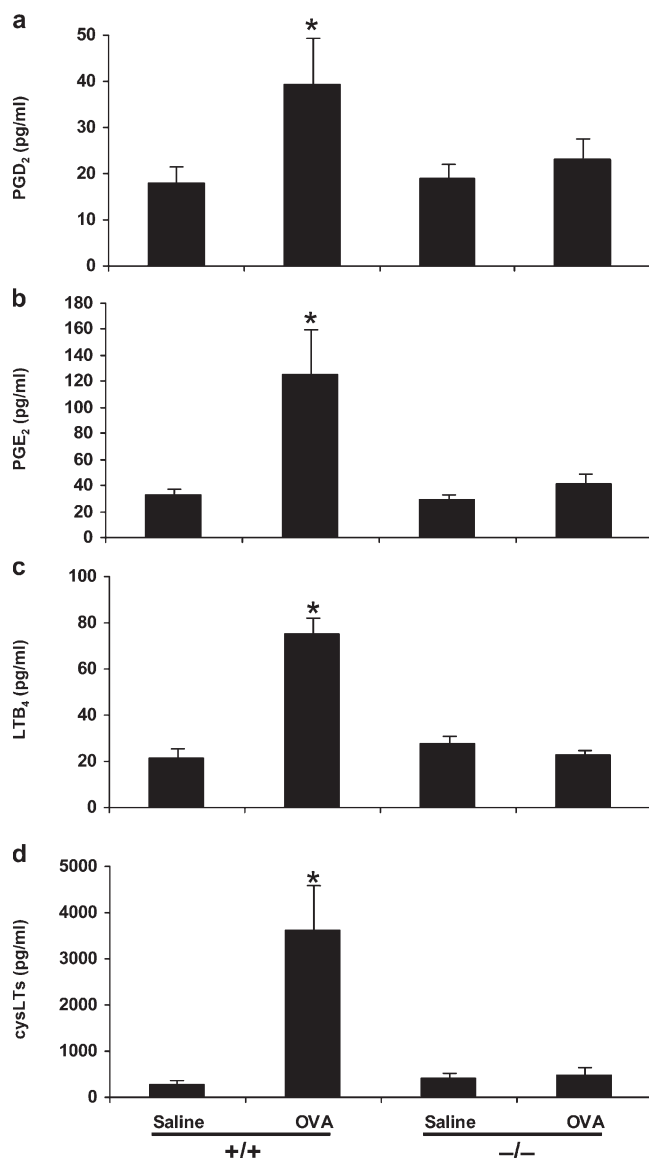


Figure 9. Impaired eicosanoid release in $sPLA_2\text{-X}^{-/-}$ mice. (a) PGD₂ (PGD₂-MOX), (b) PGE₂ (PGE₂ + 13,14-dihydro-15-keto-PGE₂ metabolite), (c) LTB₄, and (d) total cysLT levels were determined in BAL fluid obtained 1 h after the last aerosol OVA challenge on day 23 (acute asthma model) from saline- and OVA-treated $sPLA_2\text{-X}^{+/+}$ (+/+) and $sPLA_2\text{-X}^{-/-}$ (-/-) mice. Data represent the mean ± SEM. *, $P < 0.05$ versus other groups.

receptor antagonists, reduce eosinophil infiltration, mucus cell metaplasia, and collagen deposition in the airways, indicating that both LTB₄ and cysLTs C₄, D₄, and E₄ substantially contribute to the effects of IL-13 on the lung (5).

Recent studies have indicated an important role for LTB₄ and PGD₂ in directing T cells to the airways in asthma. Using mice deficient in the LTB₄ receptor BLT1, a requirement of the LTB₄-BLT1 pathway, has been demonstrated as crucial for the early recruitment of CD4⁺ and CD8⁺ T cells to the airways after allergen challenge (36). The T cell-mediated allergen-induced airway hyperresponsiveness in this model is

dependent on BLT1 and is associated with IL-13 production by the T cells (37, 38). Increased numbers of BLT1⁺ T cells are found in the airways of allergic individuals with asthma (39). Mice deficient in DP₁, one of the receptors for PGD₂, have decreased airway Th2 cytokines, eosinophil infiltration, and mucus hypersecretion compared with wild-type controls after allergen sensitization and challenge (40). PGD₂ induces preferential generation of Th2 cytokines by its interaction with the chemoattractant receptor-like molecule expressed on Th2 cells (41).

Over the past decade, it has generally been considered that cPLA₂-α is the master regulator of arachidonate release from cellular phospholipids. Early reports showed that group IIA sPLA₂ (PLA2G2A) rises to high levels in serum and synovial fluid in patients suffering from inflammatory disorders, including acute pancreatitis and rheumatoid arthritis (42, 43), but there was no evidence showing that group IIA sPLA₂ is directly responsible for the liberation of arachidonate from phospholipids for the biosynthesis of eicosanoids. More recent work has shown that sPLA₂s can augment the action of cPLA₂-α in cells to liberate arachidonate. Peritoneal macrophages from group V sPLA₂-deficient mice produce ~50% as much free arachidonate compared with wild-type cells after stimulation with zymosan, a fungal cell wall-derived agonist (14), yet there is essentially no arachidonate released in this system when macrophages from cPLA₂-α-deficient mice are used (13). Earlier studies with antisense technology to knock down the level of group V sPLA₂ also support a role for a joint action of group V sPLA₂ and cPLA₂-α in macrophages (16). Thus, cPLA₂-α and group V sPLA₂ work together by an unknown mechanism leading to maximal arachidonate release. Because both enzymes are PLA₂s that can hydrolyze arachidonyl-containing phospholipids, it is not possible at this point to know which enzyme is directly responsible for most of the phospholipid *sn*-2 lipolysis leading to arachidonate release.

In rat gastric epithelial cells (RGM1) and in rat fibroblasts (3Y1), cytokine-induced arachidonate release and PGE₂ production coincide with up-regulation and secretion of group IIA sPLA₂ into the culture medium, and studies with cPLA₂-α and sPLA₂ inhibitors support a coordinate role for both enzymes in arachidonate release (15, 17). Coordinate action of cPLA₂-α and sPLA₂s has also been recently reported in transfected cells (18, 19). There are no reports showing that endogenous sPLA₂-X is directly involved in arachidonic acid release from mammalian cells in culture. Nevertheless, we decided to explore the $sPLA_2\text{-X}^{-/-}$ mouse in an animal model of asthma because of the high activity that this enzyme shows when added to mammalian cells in culture (21, 22). The results of the present study showing a clear role for sPLA₂-X in mediating airway inflammation in a mouse model of allergic asthma are thus important and novel. Our study is the first to demonstrate an important role for an sPLA₂ in an animal model of asthma. It remains to be seen if other mammalian sPLA₂s play a role in airway inflammation and hyperreactivity.

In OVA-treated wild-type mice, a strong signal in immunohistochemical detection of sPLA₂-X in mouse lung sections was seen in airway epithelial cells and alveolar macrophages. sPLA₂-X colocalized with airway epithelial cells expressing mucin 5AC, a major gel-forming mucin of mouse (44) and human (45) airway epithelial cells. Studies using *in situ* hybridization for mRNA detection have shown that human lung epithelial cells also express sPLA₂-X, along with sPLA₂-V (24). It is generally thought that airway epithelial cells lack the lipoxygenases and cyclooxygenases needed to convert free arachidonate into leukotrienes and prostaglandins. However, *in vitro* cell co-culture studies have shown that arachidonate released from stimulated airway epithelial cells can be transferred to nearby macrophages and eosinophils for oxygenation leading to eicosanoids (46, 47). Thus, a reasonable working hypothesis for the results seen in the current study is that airway epithelial cell sPLA₂-X generates free arachidonate, which is converted into proinflammatory eicosanoids by nearby leukocytes. Studies are underway to test this hypothesis by selectively disrupting the sPLA₂-X gene in airway epithelial cells.

Our data demonstrating a profound effect of sPLA₂-X deficiency on Th2 cell-driven airway inflammation and remodeling in a mouse model of human asthma bring a new dimension to the PLA₂ field. These findings point to sPLA₂-X as a potential target for the development of novel anti-asthma drugs.

MATERIALS AND METHODS

Generation of the sPLA₂-X-deficient mouse. Embryonic stem cells (129SvEv^{Brd}-derived) containing a mutated sPLA₂-X gene were prepared by a high throughput gene trapping technique and used at Lexicon Genetics Inc. to generate a chimeric mouse in the 129SvEv^{Brd}/C57BL/6J mixed background, as previously described (48, 49). This approach uses a multiplicity of infection with virus that disfavors multiple insertions of the trapping cassette. The sPLA₂-X^{-/-} mouse was generated by breeding, and the genotype was verified by PCR using the primers LTR2, upper, and lower (primer sequences and PCR conditions are provided in Table S1, available at <http://www.jem.org/cgi/content/full/jem.20070029/DC1>). LTR2 and lower give the mutant band of 320 bp, and upper and lower give the wild-type band of 392 bp. Two lines of evidence indicate that the trapping cassette is present as a single copy in the genome: (a) rapid amplification of cDNA ends sequencing on the 3' fusion transcript revealed only a single sequence, and (b) quantitative PCR on the neomycin cassette demonstrated only a single copy in heterozygote mice.

Analysis of sPLA₂-X mRNA and protein. We used primers to exons 2 and 4 for PCR analysis of cDNA isolated from mouse tissues. Mouse testis was ground in RNA lysis buffer (600 μ l/100 mg of tissue; Promega) on ice, and RNA was isolated from 175 μ l of suspension using a kit (Z3100; Promega). A 5- μ l aliquot of the resultant 100 of RNA solution in water was converted to 20 μ l of cDNA solution using a kit (Marligen Biosciences, Inc.). 1 μ l of cDNA was used in a 25- μ l PCR reaction with primers directed at the mouse glyceraldehyde-3-phosphate dehydrogenase mRNA sequence and predicted to form a 500-bp product spanning more than one exon. From the results of agarose gel electrophoresis on 5 μ l of the PCR product after 23, 27, and 31 cycles of PCR, the amount of cDNA required just to show a perceptible band at 500 bp after 23 cycles was estimated. This amount was used in a second PCR reaction with the primers sPLA₂-X-F4 (from exon 2) and sPLA₂-X-RC (from exon 4). These primers gave an

sPLA₂-X band of the correct size (480 bp) on a 2% agarose gel after 27 cycles of PCR with cDNA derived from testis from a sPLA₂-X^{+/+} mouse, whereas 39 cycles of PCR were required to see this same band from the same amount of cDNA derived from testis from the sPLA₂-X^{-/-} mouse, corresponding to $\sim$ 1,000-fold reduction in sPLA₂-X mRNA. No PCR band was seen in a blank control after 39 PCR cycles.

To examine sPLA₂-X protein expression, stomachs from sPLA₂-X^{-/-} and sPLA₂-X^{+/+} littermate mice were flash frozen in liquid nitrogen and lyophilized. Each stomach was soaked in 200 μ l of saline, mixed by vortexing, and incubated at 4°C for 30 min. Samples were centrifuged at 16,000 g for 10 min, and the supernatants were assayed for protein by the Bradford assay and for sPLA₂-X using time-resolved immunoassay, as previously described (25).

Allergen challenge in mice. All animal use procedures were approved by the University of Washington Animal Care Committee. sPLA₂-X^{-/-} C57BL/129 mice and their wild-type sPLA₂-X^{+/+} littermates received i.p. injections of 100 μ g OVA (0.2 ml of 0.5 mg/ml; Pierce Chemical Co.) complexed with aluminum potassium sulfate (alum; Sigma-Aldrich) on days 0 and 14. Mice were anesthetized i.p. with 130 mg/kg ketamine and 8.8 mg/kg xylazine in normal saline before receiving an i.n. dose of 100 μ g OVA (0.05 ml of 2 mg/ml) on day 14, and 50 μ g OVA (0.05 ml of 1 mg/ml) on days 26, 27, and 28 (acute asthma model) or days 26, 27, 28, 47, 61, 73, 74, and 75 (chronic asthma model). Control groups received 0.2 ml of normal saline with alum i.p. on days 0 and 14, and 0.05 ml of saline without alum i.n. on days 14, 26, 27, and 28 or days 14, 26, 27, 28, 47, 61, 73, 74, and 75. Mouse plasma samples were obtained on day 29 from the OVA- and saline-treated groups and assayed for OVA-specific IgE (50). For eicosanoid analysis studies, mice were immunized i.p. with 10 μ g OVA and 1.125 mg alum in 0.2 ml of normal saline on days 0, 7, and 14 and exposed to 1% aerosolized OVA, as previously described by Myou et al. (51) for 40 min on days 21, 22, and 23. Control groups received 0.2 ml of normal saline with alum i.p. on days 0, 7, and 14, and saline by aerosol on days 21, 22, and 23.

Pulmonary function testing. On day 29, invasive pulmonary mechanics were measured in mice in response to methacholine in the same manner as previously described (3), with the following modifications: (a) the thorax was not opened; (b) mice were ventilated with a tidal volume of 200 μ l and a respiratory rate of 120 breaths per minute using a MiniVent Ventilator for Mice (Harvard Apparatus); (c) mice received aerosolized solutions of methacholine (0, 3.125, 6.25, 12.5, 25, and 50 mg/ml in normal saline) via a nebulizer aerosol system (AER 1021; Buxco Electronics) with a 2.5–4- μ m aerosol particle size generated by a nebulizer head (NEB0126; Nektar Therapeutics); and (d) a commercial plethysmography system consisting of a plethysmograph (model PLY4111), amplifier and pressure transducer system (MAX II), and Biosystem XA software (all obtained from Buxco Electronics) was used to determine R_L as calculated from the measures of pressure and flow and expressed as cm H₂O/ml/s. Noninvasive plethysmography was also assessed on day 29, as described in Supplemental materials and methods (available at <http://www.jem.org/cgi/content/full/jem.20070029/DC1>).

BAL. After tying off the left lung at the mainstem bronchus, the right lung was lavaged three times with 0.5 ml of normal saline either 1 h (for eicosanoid assays) or 24 h (for cytokine assays) after the final OVA or saline treatment. The BAL fluid was centrifuged at 250 g, and the supernatant was processed for eicosanoid assays, as described in detail in Supplemental materials and methods. Total BAL fluid cells were counted with eosinophils stained with 0.05% eosin (3), and the number of CD3⁺, CD4⁺, and CD8⁺ T cells was determined by cytofluorimetry. On day 29, alveolar macrophages were isolated from the BAL fluid cells by adherence to Esco Fluro slides (Erie Scientific Company) for 2 h in RPMI 1640 with L-glutamate (Cellgro; Mediatech, Inc.) in humidified 5% CO₂/95% air at 37°C, as previously described (52). To remove nonadherent cells, the slides were washed six times in PBS, and adherent cells were stained for sPLA₂-X expression.

Cytofluorimetry. BAL fluid cells were preincubated with 1 μ g Fc Block (anti-mouse CD16/CD32 [Fc γ III/II receptor]; BD Biosciences) per 10⁶ cells in 50 μ l of staining buffer (BD Biosciences) for 15 min at 4°C to reduce nonspecific immunostaining. After washing in PBS containing 1% FBS, pH 7.4 (Sigma-Aldrich), $\sim$ 10⁶ cells were incubated with 50 μ l PBS + 1% FBS buffer containing 1 μ g each of the following antibodies purchased from BD Biosciences: PE-anti-mouse CD3e (CD3e chain; 145-2C11), allophycocyanin-anti-mouse CD4 (L3T4; RM4-5), and FITC-anti-mouse CD8a (Ly-2; 53-6.7) for 20 min at 4°C. Cells were fixed in 1% paraformaldehyde in PBS for 16 h at 4°C, and cytofluorimetry was performed with a flow cytometry system (Cytomics FC500; Beckman Coulter).

RT-PCR. Total RNA was isolated from the right lung using an RNeasy mini kit (QIAGEN), and mRNA levels for IL-1 β , IL-2, IL-4, IL-5, IL-12 α (IL-12 p35 subunit), IL-12 β (IL-12 p40 subunit), IL-13, IFN- γ , and GAPDH were determined by RT-PCR. A PCR System (7900HT Fast Real-Time; Applied Biosystems) was used, and SYBR green PCR master mix was used (Applied Biosystems). 0.2 μ g RNA was used to synthesize first-strand cDNA with the first-strand synthesis system (SuperScript III; Invitrogen). All primers were designed using primer 3 and crossed large expanses of the intronic sequence (Table S1). PCR sizes were $\sim$ 100 bp and were confirmed by gel electrophoresis. RT-PCR readings had to be within the range of the standard curve. Sample cDNAs were diluted to 20 ng/ μ l.

Lung and spleen histopathology. The trachea, upper and lower lobes of the left lung, and spleen were fixed for 24 h in 10% neutral buffered formalin solution. The tissues were embedded in paraffin and cut into 5- μ m sections. For lung morphometry, 10 airways (0.4–0.7 mm in diameter and surrounded by smooth muscle cells) per mouse were randomly selected and examined by individuals blinded to the protocol design (3). The sections were stained with hematoxylin and eosin to evaluate airway edema (i.e., alveolar flooding by amorphous material) on a semiquantitative 0–4+ scale (0 = 0–10% alveoli with edema; 1 = 10–20% alveoli with edema; 2 = 20–30% alveoli with edema; 3 = 30–40% alveoli with edema; and 4 = 40–50% alveoli with edema) (50) and total inflammatory cell infiltration (3) on a semiquantitative scale ranging from 0–4+ and eosinophil numbers per unit of lung tissue area (2,200 μ m²). Each field, at magnification of 400 using a micrometer disc (Whipple; Bausch & Lomb), represented 2,200 μ m² of tissue area. The smooth muscle mass of the smooth muscle cell layer beneath the airway epithelial cell basement membrane was assessed on a 0–4+ scale at three sites tangential to each airway cross-section examined (4). The sections were stained with alcian blue/periodic acid Schiff (PAS) reaction to identify airway goblet cells and mucus (3). The number of goblet cells was determined as the percentage of total airway epithelial cells in each airway examined. Mucus occlusion of the airway diameter was assessed on a 0–4+ scale. Each airway section was assigned a score for airway diameter occlusion by mucus based on the following criteria: 0 = 0–10% occlusion; 1 = 10–30% occlusion; 2 = 30–60% occlusion; 3 = 60–90% occlusion; and 4 = 90–100% occlusion (3). To determine subepithelial collagen deposition/fibrosis in the airways, the sections were stained with Masson's trichrome stain (4). The intensity of the collagen deposition (0–4+ scale) was assessed in the immediate region beneath the epithelium containing the contractile elements and extracellular matrix associated with the airways, as described by Leigh et al. (53)

Immunocytochemistry. sPLA₂-X expression in mouse lung and spleen was localized by immunocytochemistry using light microscopy (54). Tissues were fixed in methyl Carnoy's solution and embedded in paraffin, and 5- μ m sections were cut, placed on slides (Super Frost Plus; VWR Scientific), deparaffinized, hydrated, and rinsed in PBS. All immunolabeling procedures were performed at 24°C. Endogenous peroxidase was inactivated by incubating the tissue sections in 0.75% hydrogen peroxide for 30 min. After rinsing in PBS, the sections were incubated with the primary antibody, polyclonal rabbit anti-sPLA₂-X-specific antisera (55), at a 1:50 dilution for 25 min, followed by rinsing in PBS and incubation with the secondary antibody, goat anti-rabbit antibody conjugated to horseradish peroxidase (Vector Laboratories), at a

1:20 dilution for 25 min. The controls were either PBS or normal rabbit IgG (Vector Laboratories) used in place of the primary antibody. After rinsing in PBS, the sections were incubated with 0.5% 3',3'-diaminobenzidine tetra-chloride (Sigma-Aldrich) in PBS and 0.15% hydrogen peroxide for 15 min for detection of peroxidase. The sections were rinsed in PBS, and nuclei were counterstained with 1% methyl green in distilled water for 3 min. The sections were dehydrated in ethanol, cleared in xylene, and mounted on glass slides with Permount (Fisher Scientific). BAL fluid alveolar macrophages, isolated by overnight adherence to microscope slides, were also stained for sPLA₂-X by immunocytochemistry.

Immunofluorescence confocal microscopy. For immunofluorescence double staining of mucin 5AC and sPLA₂-X, lung tissue was frozen in liquid nitrogen immediately after collection, embedded in OCT compound (Tissue-Tek; Sakura Finetek USA, Inc.), and cut into 10- μ m-thick cryostat sections. After rinsing in PBS, the slides were fixed in acetone for 5 min, washed three times in PBS, and blocked in 2% dry milk for 30 min at room temperature. The sections were incubated with the primary antibodies (both at a dilution of 1:100 in PBS), rabbit anti-mouse sPLA₂-X (55) and goat anti-mouse mucin 5AC (K-20; Santa Cruz Biotechnology, Inc.), for 40 min at room temperature, washed three times in PBS, and incubated with the secondary antibodies (both at a dilution of 1:100 in PBS; Invitrogen), Alexa Fluor 594 (i.e., red fluorophore) donkey anti-rabbit IgG and Alexa Fluor 488 (i.e., green fluorophore) donkey anti-goat IgG, for 30 min at room temperature. After washing in PBS, coverslips were placed with IMMU-MOUNT (Shandon Inc.), and slides were stored in the dark at 4°C. Sections were examined on a confocal microscope (LSM510Meta; Carl Zeiss MicroImaging, Inc.) running software version 4.0. Fluorescence excitation was provided using an argon laser line (488 nm) and a helium-neon laser line (543 nm).

Cytokine and eicosanoid analyses. Lincoplex multiplexed biomarker immunoassays of BAL fluid for IL-1 β , IL-2, IL-4, IL-5, IL-12 (p70), and IFN- γ were performed using Luminex instrumentation (3.2 pg/ml detection limit; LINCOP Research) according to the manufacturer's instructions. IL-13 (1.5 pg/ml detection limit; R&D Systems), LTB₄ (13 pg/ml detection limit; Cayman Chemical), cysLTs (LTC₄/LTD₄/LTE₄, 13 pg/ml detection limit; Cayman Chemical), PGD₂ (measured as the stable MOX derivative PGD₂-MOX after treatment of the samples with methoxy-lamine hydrochloride; 3.1 pg/ml detection limit using a PGD₂-MOX enzyme immunoassay (EIA) kit; Cayman Chemical), and PGE₂ (measured as the total of PGE₂ [15 pg/ml detection limit using a PGE₂ kit—monoclonal; Cayman Chemical] and its 13,14-dihydro-15-keto metabolite [2 pg/ml detection limit using a PGE₂ metabolite EIA kit; Cayman Chemical]) were determined by EIA. The fraction of cysLTs that are LTC₄, LTD₄, and LTE₄, was determined by combined liquid chromatography–electrospray tandem mass spectrometry using a modification of the method of Kita et al. (56). Full details of eicosanoid analyses are provided in Supplemental materials and methods.

For flow cytometric analysis of intracellular cytokines, total splenic cell populations from experimental and control mice were plated in 24-well plates (4 $\times$ 10⁶ cells per well) and activated with 200 μ g/ml OVA (Pierce Chemical Co.) overnight. The samples were stained with pericinin-chlorophyll-*a*-protein-conjugated anti-CD4, allophycocyanin-conjugated anti-CD8, and PE-conjugated antibodies against the following cytokines: IL-4, IL-5, IL-10, TNF- α , and IFN- γ (antibodies were obtained from BD Biosciences and eBioscience). The intracellular staining for cytokines was performed using the manufacturer's standard protocol (BD Biosciences). Samples were analyzed using a flow cytometer (FACSCanto; BD Biosciences), and the data were analyzed using FlowJo software (TreeStar Inc.).

For enzyme-linked IL-4 and IFN- γ immunospot (ELISPOT) assays (BD Biosciences), total spleen cells from OVA- and saline-treated sPLA₂-X^{-/-} mice and wild-type controls were collected, plated in 96-well plates (2 $\times$ 10⁵ cells/well), and incubated in triplicate in 5% CO₂/95% air at 37°C in the absence or presence of 5 ng/ml PMA (Sigma-Aldrich) and 500 ng/ml ionomycin (Sigma-Aldrich) for 24 h or 500 μ g/ml OVA (Pierce Chemical Co.) for

5 d according to the manufacturer's protocol (BD Biosciences). CD4⁺ T cells were isolated from the spleen cells using Dynabeads Mouse CD4 (L3T4) and DETACHsBEAD Mouse CD4 (Dyna), according to the manufacturer's instructions. 4×10^4 CD4⁺ T cells/well were incubated in triplicate in 5% CO₂/95% air at 37°C in the absence or presence of 1 µg/ml of hamster anti-mouse CD3e/2 µg/ml of hamster anti-mouse CD28 monoclonal antibodies (BD Biosciences) for 5 d, and IL-4 immunospot was performed.

Statistical analysis. Data are reported as the mean $\pm$ SEM. Differences were analyzed for significance ($P < 0.05$) by analysis of variance using the least significant difference method.

Online supplemental material. Fig. S1 shows sPLA₂-X expression in airway cells of OVA-treated sPLA₂-X^{+/+} mice by immunocytochemistry. Fig. S2 shows that sPLA₂-X expression is seen in splenic mononuclear cells in sPLA₂-X^{+/+} mice but is absent in sPLA₂-X^{-/-} mice by immunocytochemistry. Fig. S3 shows decreased goblet cell metaplasia and mucus release in the lungs of sPLA₂-X^{-/-} mice after allergen challenge in an acute asthma model. Fig. S4 shows reduced levels of eosinophils in BAL fluid of OVA-treated sPLA₂-X^{-/-} mice in a chronic asthma model. Fig. S5 shows reduced allergen-induced airway inflammatory cell infiltration and edema in sPLA₂-X^{-/-} mice in a chronic asthma model. Table S1 provides the sequence of PCR primers and PCR conditions used. Supplemental materials and methods contains details of the procedure for BAL fluid eicosanoid analysis by liquid chromatography–electrospray tandem mass spectrometry and EIA and pulmonary function testing by noninvasive plethysmography. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20070029/DC1>.

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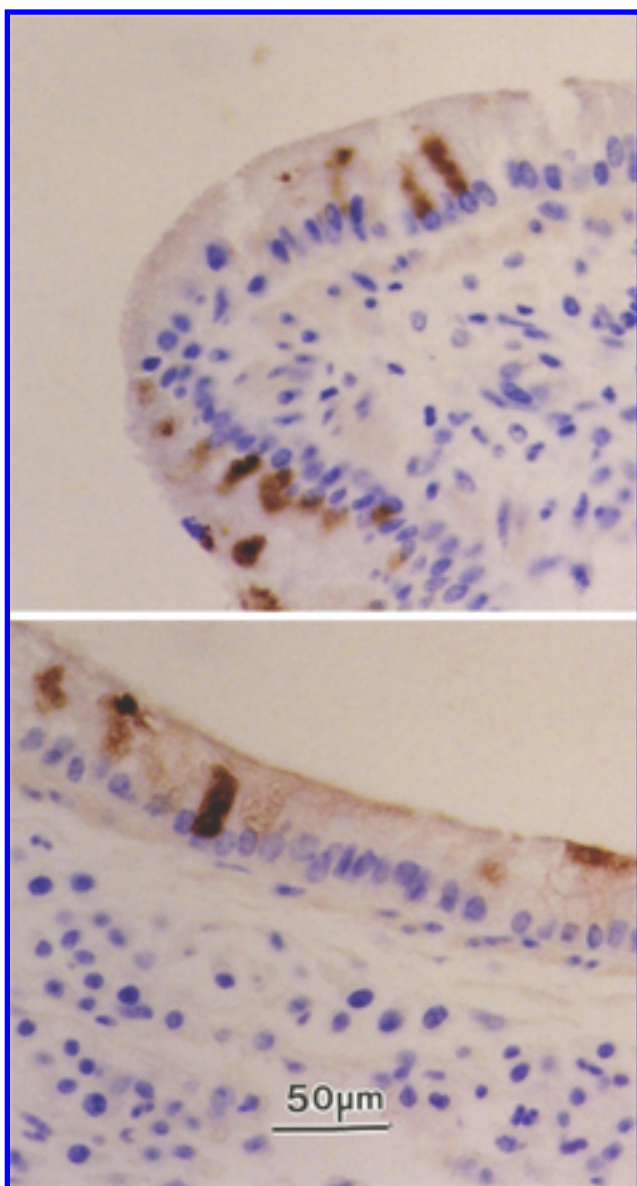


Figure S1. sPLA₂-X expression in airways of wild-type mice. Lung tissue was obtained on day 29 (acute asthma model) from sPLA₂-X^{+/+} mice treated with OVA and examined by immunocytochemistry for sPLA₂-X expression (top and bottom). Bar, 50 µm.

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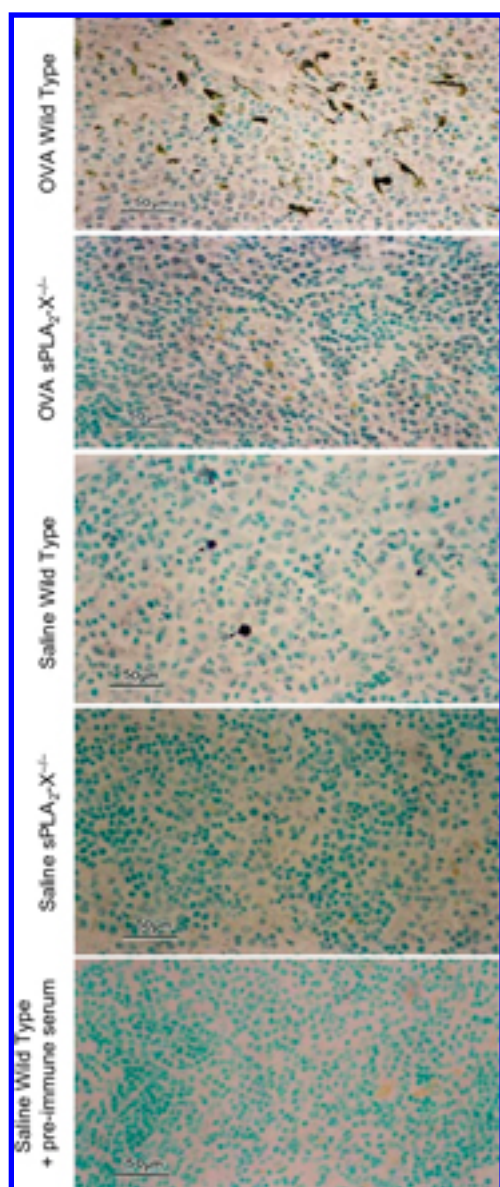
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Figure S2. Absent sPLA₂-X expression in the spleens of sPLA₂-X^{-/-} mice. Splenic tissue was obtained on day 29 from OVA- and saline-treated sPLA₂-X^{+/+} and sPLA₂-X^{-/-} mice. Sections were examined for sPLA₂-X expression by immunocytochemistry, as described in Materials and methods. As a control, preimmune antibody was used in place of the anti-sPLA₂-X-specific antisera shown (bottom). Arrows indicate sPLA₂-X-positive cells. Bars, 50 µm.

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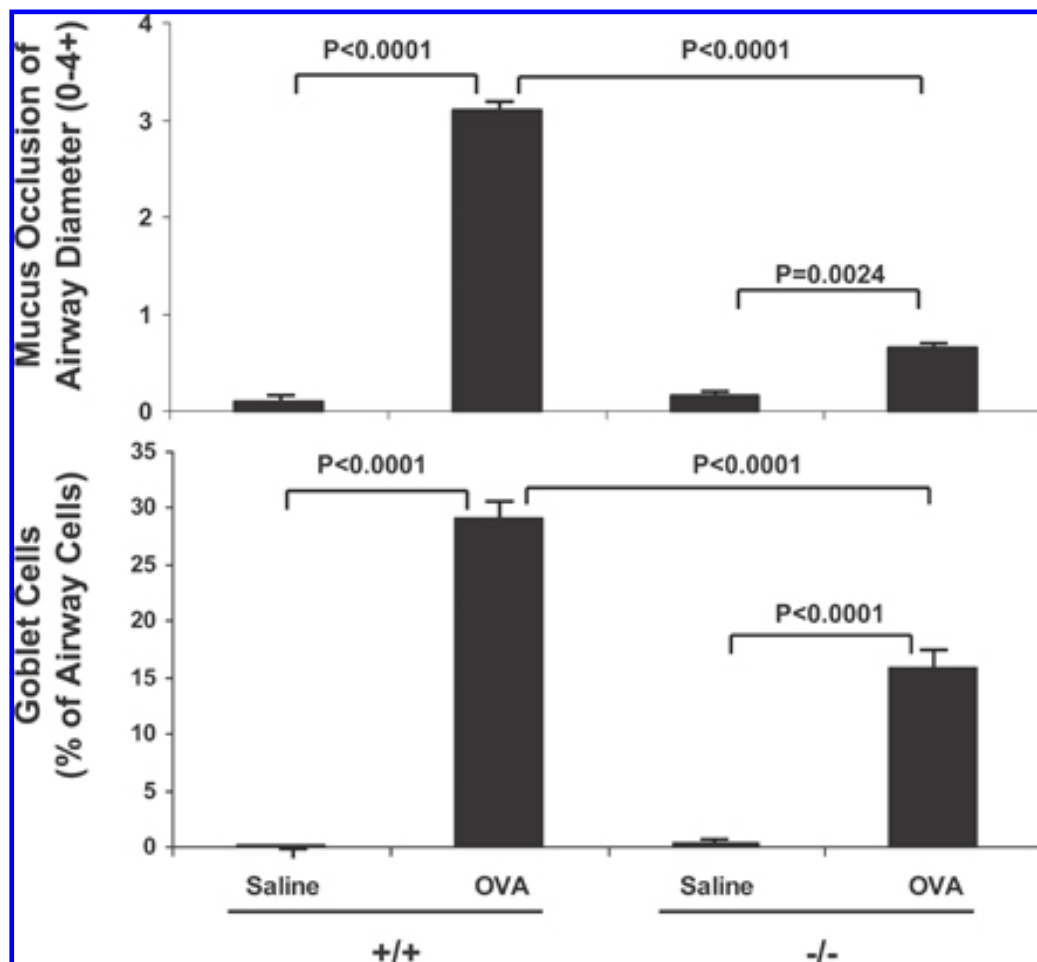
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Figure S3. Impaired allergen-induced airway goblet cell metaplasia and mucus hypersecretion in sPLA₂-X^{-/-} mice. Lung sections of saline- and OVA-treated sPLA₂-X^{+/+} and sPLA₂-X^{-/-} mice obtained on day 29 were stained with alcian blue/PAS, and the occlusion of airway diameter by mucus (0-4+ scale) and the percentage of total airway epithelial cells positive for mucus glycoproteins were determined by morphometry ($n = 4$ for each group). Data represent the mean $\pm$ SEM.

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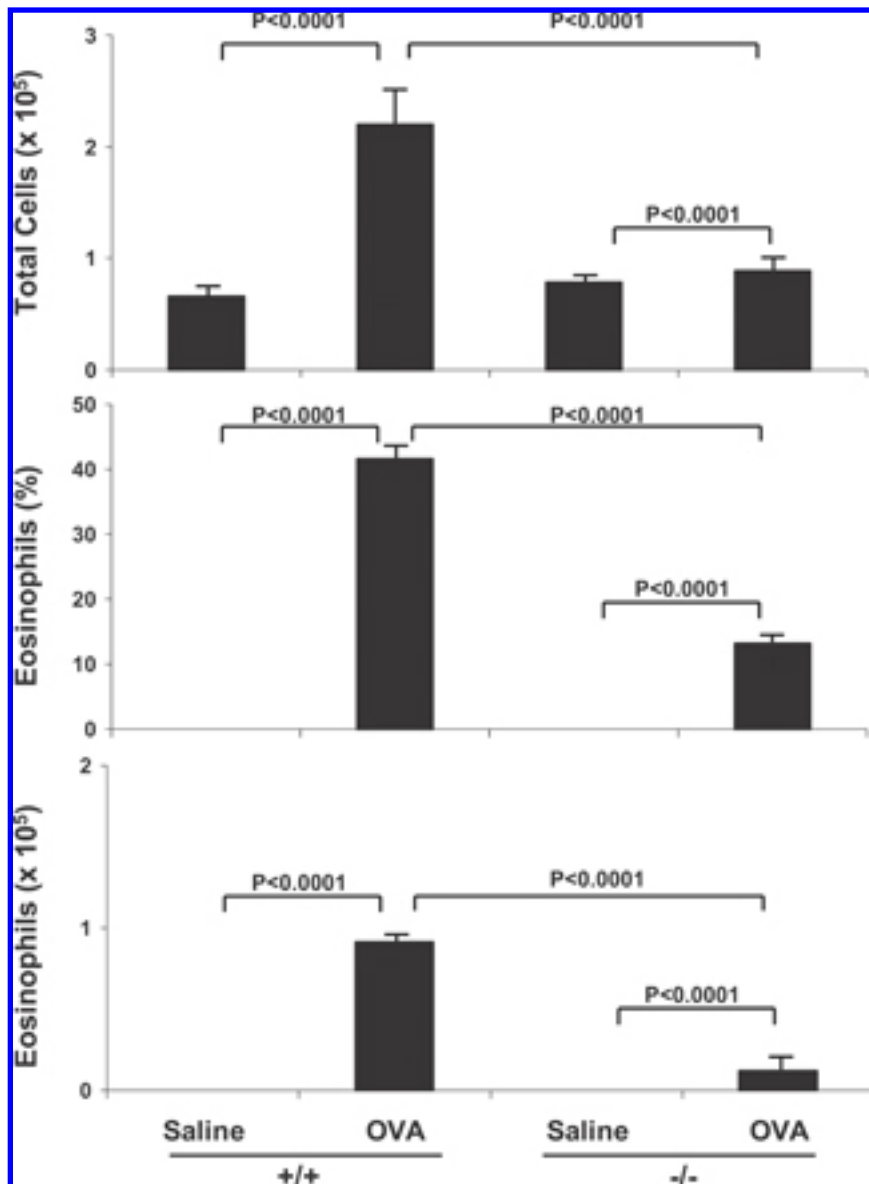


Figure S4. Impaired allergen-induced eosinophilia in BAL fluid in $sPLA_2-X^{-/-}$ mice. BAL fluid was obtained on day 76 from $sPLA_2-X^{+/+}$ (+/+) and $sPLA_2-X^{-/-}$ (-/-) mice treated with either saline or OVA, and the number of total cells and the percentage and number of eosinophils were determined. ($n = 4$ for each group). Data represent the mean $\pm$ SEM.

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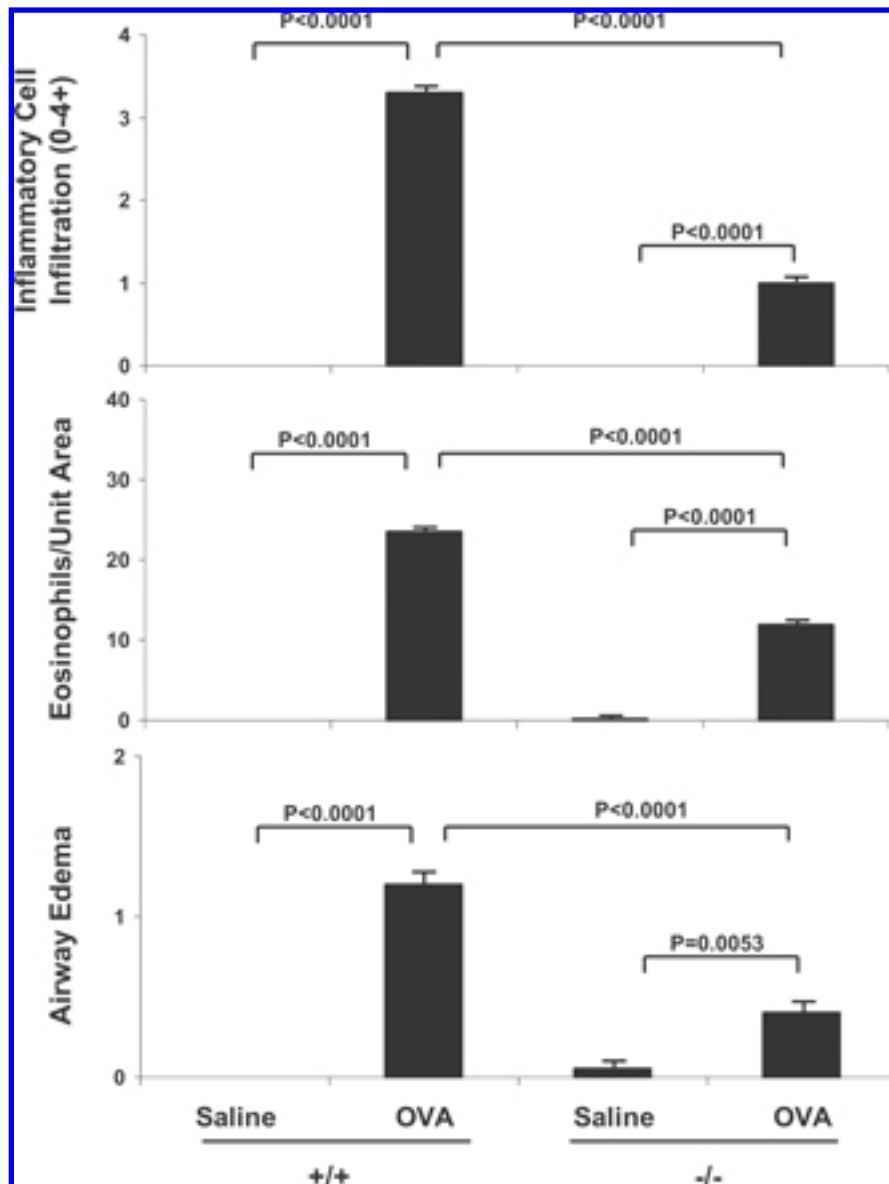
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Figure S5. Impaired allergen-induced airway inflammatory cell infiltration and edema in *sPLA₂-X^{-/-}* mice. Lung tissue was obtained on day 76 from *sPLA₂-X^{+/+}* (+/+) and *sPLA₂-X^{-/-}* (-/-) mice treated with either saline or OVA, and lung sections were stained with hematoxylin and eosin. The intensity of the total inflammatory cell infiltrate (0-4+ scale), the number of eosinophils per unit area (2,200 μm^2), and airway edema (0-4+ scale) were determined by morphometry. ($n = 4$ for each group). Data represent the mean $\pm$ SEM.

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Table S1. • Sequence of PCR primers and PCR conditions

Experiment	Primer name and sequence (5' to 3')	PCR conditions
sPLA ₂ -X genotyping	LTR2: AAATGGCGTTACTTAAGCTAGCTTGC Upper: CCCTCCCCTTTAGTGTGCATGTT Lower: CAGTCAGCCTGGGCTATGGTGTG	94°C for 45 sec, then 35 cycles at 64°C for 1 min/72°C for 1 min + 10-sec autoextension, then 72°C for 10 min
sPLA ₂ -X PCR	sPLA ₂ -X-F4: gagctggcagggaccttgattgtgtg sPLA ₂ -X-RC: gaagtgcgtttccatgtgcaaagggac	95°C for 15 min, then multiple (23–39) cycles at 94°C for 45 sec/60°C for 1 min/68°C for 45 sec, then 68°C for 5 min
GAPDH PCR	cagaacatcatccctgcatccactggtg gaaattgtgagggagatgctcagtgtg	same as for sPLA ₂ -X PCR
IL-1 β RT-PCR	TGAAGCAGCTATGGCAACTG GGGTCCGTCAACTTCAAAGA	50°C for 2 min; 95°C for 10 min, then 50 cycles at 95°C for 15 sec/60°C for 1 min
IL-2 RT-PCR	AACCTGAAACTCCCCAGGAT CGCAGAGGTCCAAGTTCATC	same as for IL-1 β RT-PC
IL-4 RT-PCR	GGCATTTTGAACGAGGTCAC AAATATGCGAAGCACCTTGG	same as for IL-1 β RT-PC
IL-5 RT-PCR	ATGGAGATTCCCATGAGCAC AGCCCCTGAAAGATTTCTCC	same as for IL-1 β RT-PC

IL-12 α RT-PCR	GCCAGGTGTCTTAGCCAGTC TGATCGATGTCTTCAGCAGTG	same as for IL-1 β RT-PC
IL-12 β RT-PCR	ATCGTTTTGCTGGTGTCTCC CATCTTCTTCAGGCGTGTC	same as for IL-1 β RT-PC
IL-13 RT-PCR	CCTCTGACCCTTAAGGAGCTT ATGTTGGTCAGGGAATCCAG	same as for IL-1 β RT-PC
IFN γ RT-PCR	GCGTCATTGAATCACACCTG TGAGCTCATTGAATGCTTGG	same as for IL-1 β RT-PC
GAPDH RT-PCR	CGTCCCGTAGACAAAATGGT TCAATGAAGGGGTCGTTGAT	same as for IL-1 β RT-PC

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Henderson et al., <http://www.jem.org/cgi/content/full/jem.20070029/DC1>

SUPPLEMENTAL MATERIALS AND METHODS

Analysis of BAL fluid eicosanoids by high performance liquid chromatography (HPLC)–tandem mass spectrometry. After collection of 0.9–1.4 ml of BAL fluid, it was immediately centrifuged at 1,200 rpm for 5 min at 4°C in a swinging bucket rotor at room temperature to pellet BAL fluid cells. A 0.5-ml portion of supernatant was immediately transferred to a labeled, amber glass vial containing 2 ml methanol/0.2% formic acid (HPLC-grade methanol and reagent-grade formic acid) and 10 µl of internal standard mixture. The deuterated internal standards were as follows: 50 pg/µl each of 5(S)-HETE-d8, LTB₄-d4, PGE₂-d4, PGD₂-d4, 6-keto-PGF_{1α}-d4, and TxB₂-d4; 100 pg/µl PAF-C₁₆-d4; and 20 pg/µl each of LTC₄-d4 and LTE₄-d4, (all obtained from Cayman Chemical except the labeled LTC₄ and LTE₄, which were prepared by in-house synthesis and stored as stock solution in methanol/0.2% formic acid at –80°C under argon). The sample in the vial was mixed briefly on a vortexer, and the vial was capped with a Teflon-line septum screw cap and placed on dry ice and then into a –80°C freezer.

After BAL collection, samples in the vials were thawed on ice, and the liquid was transferred to a 16 × 125-mm glass tube. Samples were brought to ~15% methanol by addition of Milli-Q (Millipore) purified water/0.03% formic acid. Samples were loaded using a Pasteur pipet onto an Oasis HLB column (10-mg column bed; Waters Corporation) that was previously equilibrated with 1 ml methanol/0.2% formic acid, followed by 2 × 1 ml water/0.03% formic acid. A 1-ml portion of water/0.03% formic acid was added to the empty culture tube, and the sample was mixed briefly on a vortexer. The liquid was transferred to the Oasis HLB cartridge. The columns were processed in a vacuum manifold (Waters Corporation) connected to a water aspirator. The receiver tubes in the manifold were replaced with amber autosampler vials without inserts (Agilent Technologies). The Oasis cartridge was removed from the manifold so that traces of water in the column tip could be pushed out of the column by applying a positive pressure of N₂ to the top of the column using Tygon tubing. N₂ was also used to clear any water remaining in the transfer tube adaptor attached to the column tip in the manifold. Eicosanoids were eluted from the cartridge into the autosampler vial with 100 µl methanol/0.2% formic acid, followed by 2 × 200 µl methanol/0.2% formic acid. The volume in each autosampler vial was reduced to 200–250 µl with a gentle stream of N₂. The liquid was transferred to an autosampler insert (Agilent Technologies) with a Pasteur pipet. The volume in the insert was reduced to ~50 µl with N₂, the vial was capped with a PTFE/silicone septum cap (Agilent Technologies), and the vial was purged with argon and stored at –80°C until submitted to HPLC–tandem mass spectrometry (within 1–2 d).

Combined HPLC–tandem mass spectrometry was performed using a triple-quadrupole mass spectrometer with an electrospray ionization source (API-4000; Applied Biosystems). Mass spectrometer parameters were as described previously by Kita et al. (1). The HPLC column was a Shiseido Capcell PAK C18 MGII S3 column (1 id × 75 mm; ESA Biosciences, Inc.). The column temperature was 46°C, and the flow rate was 0.12 ml/min. The column was preequilibrated with 70% solvent A (water)/30% solvent B (CH₃CN/0.1% formic acid) for 12 min. After injection of 5 µl of sample, the solvent program was as follows: 3 min, 70% A; 3–10 min, 35% A; and 10–13 min, 0% A. Ion peaks corresponding to each analyte and internal standard were integrated, and the amount of each analyte was obtained from the area ratio and the amount of internal standard added to each sample. It was assumed that LTD₄ and LTE₄ give the same response in the mass spectrometer as does LTC₄ (only an internal standard for LTC₄ was present). The analyte amounts were corrected for the amount of BAL fluid obtained from each mouse to obtain the amount of eicosanoid per mouse lung.

Analysis of the BAL fluids PGE₂, LTB₄, and cysLT by EIA. A 0.25-ml aliquot of the BAL fluid supernatant (after centrifugation of the collected BAL fluid; see Analysis of BAL fluid...) was mixed with 0.5 ml methanol/0.2% formic acid (internal standards were omitted). The sample was processed using an Oasis HLB cartridge as described in Analysis of BAL fluid... After elution of eicosanoids, the 500-µl sample was placed in a centrifugal vacuum concentrator (Savant SpeedVac Concentrator; Fisher Scientific) under vacuum for 30 min at room temperature to remove all traces of methanol. The residue was resuspended in 20 µl EIA buffer (Cayman Chemical), and portions were used for EIA analysis (i.e., PGE₂ kit–monoclonal, PGE₂ metabolite EIA kit, LTB₄ EIA kit, and cysLT EIA kit from Cayman Chemical; see Materials and methods) according to the manufacturer's instructions.

Analysis of the BAL fluid PGD₂ by EIA. A 0.25-ml aliquot of the BAL fluid supernatant (after centrifugation of the collected BAL fluid; see Analysis of BAL fluid...) was mixed with 0.25 ml of ice-cold, reagent-grade acetone in a glass vial. After incubation on ice for 5 min, the sample was centrifuged at 3,000 *g* for 10 min at 4°C. The supernatant was transferred to a new glass vial on ice. After resuspension of the pellet in 0.5 ml of ice-cold acetone, the sample was briefly mixed on a vortexer and centrifuged as in Analysis of BAL fluid... After combining the supernatants, the sample was stored on dry ice for up to 4–5 h. The sample was concentrated to dryness in a Savant SpeedVac concentrator at room temperature, and 100 µl EIA buffer from the PGD₂-MOX EIA kit (Cayman Chemical) was added to resuspend the dried sample. The sample was submit-

ted to methoximation as indicated in the Cayman instructions. Two volumes of methanol/0.2% formic acid were added, and the pH was checked by spotting an ~1-ml aliquot onto pH paper to ensure that the pH was <4. The sample was diluted with water/0.03% formic acid to give 15% methanol. The sample was submitted to purification using an Oasis HLB cartridge as in Analysis of BAL fluid... The methanol eluant was dried in the Savant SpeedVac concentrator under vacuum for 30 min at room temperature to ensure that all methanol was removed. The residue was dissolved in 20 µl of Cayman EIA buffer before EIA analysis according to the manufacturer's instructions.

Noninvasive plethysmography. For noninvasive plethysmography, mice were challenged with increasing doses of aerosolized methacholine (0, 5, and 20 mg/ml in normal saline) generated by an ultrasonic nebulizer (DeVilbiss Health Care, Inc.) for 2 min. The degree of bronchoconstriction was expressed as enhanced pause (Penh), a calculated dimensionless value that correlates with measurement of airway resistance, impedance, and intrapleural pressure (2, 3). *Penh* is calculated as follows: $Penh = [(T_e/T_r - 1) \times (PEF/PIF)]$, where T_e is expiration time, T_r is relaxation time, PEF is peak expiratory flow, and PIF is peak inspiratory flow. T_r is the time it takes for a user-defined percentage of the expired volume (computed from the box-flow waveform) to be exhaled, starting at end expiration. The endpoint of T_r is chosen to be when 40% of the volume remains to be exhaled (4). *Penh* is primarily independent of functional residual capacity, tidal volume, and respiratory rate, because the ratio of measurements is obtained during the same breath and has a strong correlation with both airway resistance (R_{aw}) measured directly in anesthetized, tracheotomized, and mechanically ventilated mice (2, 3, 5) and the intensity of the allergen-induced airway eosinophil infiltration in the mouse asthma model (6).

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