

Mice Lacking 12/15-Lipoxygenase Have Attenuated Airway Allergic Inflammation and Remodeling

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Arachidonate 15-lipoxygenase (LO)-1 has been implicated in allergic inflammation and asthma. The overall effect of 15-LO in allergic inflammation *in vivo* is, however, unclear. This study investigates systemic allergen sensitization and local allergic airway inflammation and remodeling in mice lacking the murine 12/15-LO, the ortholog to human 15-LO-1. Upon systemic sensitization with intraperitoneal ovalbumin, 12/15-LO^{-/-} mice produced elevated levels of allergen-specific immunoglobulin E compared with wild-type (Wt) controls. However, when challenged with repeated aerosolized allergen, sensitized 12/15-LO^{-/-} mice had an impaired development of airway allergic inflammation compared with Wt controls, as indicated by reduced bronchoalveolar lavage fluid leukocytes (eosinophils, lymphocytes, macrophages) and Th₂ cytokines (IL-4, IL-5, IL-13), as well as tissue eosinophils. Allergen-induced airway epithelial proliferation was also significantly attenuated in 12/15-LO^{-/-} mice, whereas goblet cell hyperplasia was unaffected. However, 12/15-LO^{-/-} mice had significantly reduced luminal mucus secretions compared with Wt controls. The repeated allergen challenges resulted in a dramatic increase of α -smooth muscle actin-positive alveolar cells in the peripheral airways, a phenomenon that was significantly less developed in 12/15-LO^{-/-} mice. In conclusion, our data suggest that 12/15-LO^{-/-} mice, although having a fully developed systemic sensitization, did not establish a fully developed allergic airway inflammation and associated manifestations of central and peripheral airway remodeling. These data suggest that 12/15-LO-derived metabolites play an important pathophysiologic role in allergen-induced inflammation and remodeling. Hence, pharmacologic targeting of the human 15-LO-1 may represent an attractive therapeutic strategy to control inflammation and remodeling in asthma.

Keywords: allergy; 15-LO; asthma; remodeling

Lipid-derived mediators are important players in inflammatory diseases like asthma. The cells involved in their synthesis contain a family of structurally related enzymes that catalyze the metabolism of arachidonic acid, released from phospholipids by inflammatory stimuli, to biologically active compounds. The enzyme 5-lipoxygenase (5-LO) catalyzes the conversion of arachidonic acid to leukotrienes, which are proinflammatory and potent bronchoconstrictors (1). Several studies also indicate a role in asthma of 15-LO-1, a lipoxygenase with a narrow tissue distribution but which is expressed in many of the cells implicated in asthma pathology (e.g., airway epithelial cells, eosinophils,

CLINICAL RELEVANCE

This study shows that while 12/15-lipoxygenase (LO) is not needed for allergen sensitization, it is crucial for manifestation of allergen-induced inflammation and remodeling. Hence, targeting 15-LO-1 represents an attractive therapeutic strategy in asthma control.

macrophages, dendritic cells, and mast cells [2–6]). Apart from metabolizing free arachidonic acid, 15-LO-1 can also oxygenate fatty acids attached to membrane phospholipids (7), and these oxidated phospholipids might contribute to 15-LO-1 signaling in inflammation (8). The Th₂ cytokines IL-4 and IL-13 induce the expression of 15-LO-1 in cultured mast cells (6), monocytes (9), and airway epithelial cells (10), and in monocytes this induction could be inhibited by IFN- γ and glucocorticoids (9).

The amount of 15-LO-1 is significantly increased in the bronchial tissue of patients with asthma or chronic bronchitis compared with control subjects (11, 12). Smokers, in particular smokers with chronic bronchitis, also display an increased expression of 15-LO-1 in bronchial biopsies (13). The 15-LO-1-derived metabolite 15-hydroxy-eicosatetraenoic acid (15-HETE) has been used as a biomarker for 15-LO-1 activity in various cells and tissues, and 15-HETE is the major metabolite from arachidonic acid in human tracheal epithelial cells, human bronchi, human lung homogenate, and nasal polyps (7, 14). Increased levels of 15-HETE have also been observed in bronchoalveolar lavage (BAL) and sputum samples in both mild and severe asthma (12, 15, 16).

While the examples above suggest a potential involvement of 15-LO in asthma, the functional consequences of 15-LO activity *in vivo* are complex. In addition to the proinflammatory properties ascribed to certain 15-LO-1-derived products (5, 7, 17), antiinflammatory lipoxins can also partly be formed via the 15-LO pathway (18). Thus, in diseases like allergic asthma, 15-LO activity may both worsen and improve the underlying airway inflammation. Taking the complex and multiple roles of 15-LO-1 into consideration, the aim of the present study was to explore the overall functional consequences of this type of enzyme in an *in vivo* model of allergic inflammation. For this purpose we have used knock-out mice lacking 12/15-LO, the ortholog to human 15-LO-1. There are many similarities between human 15-LO-1 and animal 12/15-LO (7). The expression pattern of human 15-LO-1 and murine 12/15-LO is similar (19), and the cytokines IL-4 and IL-13 induce the expression of 12/15-LO in both murine and human macrophages (5). Furthermore, allergen challenge up-regulates the expression in airway epithelial cells of 12/15-LO in mice (20) and 15-LO-1 in humans (11, 12).

In our aim to explore the net effect *in vivo* between proinflammatory and antiinflammatory properties of 12/15-LO, the focus in this study has been on three key phases of an airway allergic inflammation: (1) the systemic sensitization to allergen, (2) the subsequent development of a airway cellular inflammation

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upon allergen inhalation, and (3) early manifestations of inflammation-induced structural alterations within the airways (i.e., airway remodeling).

MATERIALS AND METHODS

Animals

The capacity to develop systemic allergen sensitization as well as local airway allergic inflammation was tested in wild-type (Wt) mice (C57/BL6) and in age- and sex-matched mice that carry targeted 12/15-LO^{-/-} alleles on a C57BL/6 background (21) (12/15-LO^{-/-} mice, Alox 15; Jackson Laboratory, Bar Harbor, ME). All animals were kept in a modern barrier facility with a 12-hour light-dark cycle and were allowed water and food *ad libitum*. The animal procedures were approved by the local ethics committee (Lund/Malmö, Sweden).

Systemic Immunizations

All mice were actively immunized against ovalbumin (OVA, grade III; Sigma, St. Louis, MO) at Day 1 by an intraperitoneal injection (7.5 μ g OVA + 1.5 mg AlOH₃) (22).

Systemic Sensitization: Plasma Levels of Allergen-Specific Immunoglobulin E

An identical booster injection was also given at Day 7 to separate mice ($n = 6-7$ animals/group) to assess plasma levels of allergen-specific immunoglobulin (Ig)E (as a parameter of systemic sensitization) (23). At Day 21 after the initial intraperitoneal sensitization, animals were killed and systemic blood was collected. Plasma supernatants were stored for determination of OVA-specific IgE (22). Briefly, heparinized blood from immunized animals was centrifuged and plasma was collected and stored at -70°C until analysis. To determine plasma levels of allergen (OVA)-specific IgE, 96-well flat-bottomed plates (Nunc, Roskilde, Denmark) were incubated overnight with OVA (Sigma) at a concentration of 100 μ g/ml, followed by incubation with 3% BSA. Mouse plasma was serially diluted, followed by application of biotin-conjugated rat anti-mouse IgE (R35-72; BD Biosciences Pharmingen, San Jose, CA). Bound R35-72 was detected with horseradish peroxidase-conjugated streptavidin (Sigma). Plates were developed for 30 minutes using TMB peroxidase substrate (KPL, Gaithersburg, MD) and read at 450 nm. A plasma pool from OVA-sensitized Wt mice was used as an internal laboratory standard. The titer of allergen-specific antibodies was expressed as arbitrary units/ml plasma.

Assessments of Airway Inflammation and Remodeling after Local Airway Allergen Challenge

Challenge and termination. At Days 21–24 after one single intraperitoneal immunization (Day 1), the animals were exposed to daily allergen provocations (30 min inhalation of 1% aerosolized OVA). The aerosol was generated by a micronebulizer (Bird, 500 ml, Inline micronebulizer; Bird Co., Palm Springs, CA) using an air pressure of 4 bar. Animals were killed 8 hours after the final allergen challenge by an intraperitoneal overdose of pentobarbital sodium. BAL samples were collected for determination of luminal cells and cytokines (*see below*). The main bronchi and lung tissue samples were excised and immediately immersed in fixative for histologic analysis (*see below*). Samples for paraffin histochemistry were immersed in buffered 4% paraformaldehyde, dehydrated through a series of increasing EtOH solutions, and embedded in paraffin. Samples for cryohistochemistry were immersed overnight in Stefanini's fixative (PBS buffer supplemented with 3% formaldehyde and 1% picric acid) at 4°C , rinsed in PBS buffer supplemented with 10% sucrose, and embedded in O.C.T. freeze compound (Tissue-Tek, Sakura Finetek, Zoeterwoude, NL).

BAL. Bronchoalveolar lavage was performed by cannulation of the trachea via a midcervical incision, and 1 ml PBS was introduced to the lungs during 2 minutes using 10 cm H₂O filling pressure (22, 24). The lavage fluid was centrifuged (10 min, 4°C , $160 \times g$) and the supernatant stored at -70°C for subsequent cytokine analysis. Total numbers of BAL cells was determined using a hemocytometer. Differential cell counts were obtained from May-Grunwald-stained cytospin slides (Shandon, Runcorn, UK). Neutrophils, lymphocytes, eosinophils, and large mononuclear cells (i.e., monocytes and alveolar macrophages)

were identified by morphology and staining characteristics. Macrophage-like cells were subdivided into mature alveolar macrophages (large, often irregular, cells with characteristic bleb-like structures in their cytoplasm) and monocytes/immature macrophages (large mononucleated cells with less abundant and bleb-free cytoplasm).

Cytokine levels in BAL fluid. Th2 cytokines in the BAL fluid supernatant were determined by commercially available ELISA kits according to the instructions provided by the manufacturer (Endogen Inc., Woburn, MA, for IL-4; R&D Systems Inc., Minneapolis, MN, for IL-5 and IL-13). Detection limits were 5 pg/ml for IL-4, 7 pg/ml for IL-5, and 1.5 pg/ml for IL-13 (24).

Eosinophil peroxidase staining. The distribution of eosinophils was assessed in 8- μ m cryosections by histochemical staining of cyanide-resistant eosinophil peroxidase (EPO) (24, 25). Cryosections were exposed to 100 μ l incubation solution (PBS-buffer pH 7.6, containing 3.3 diaminobenzidine tetrahydrochloride [75 mg/100 ml; Sigma], H₂O₂ [100 μ l/100 ml], and NaCN [50 mg/100 ml]) for 7 minutes at room temperature. After rinsing in tap water, samples were mounted in Kaiser's medium. Lung sections with representative bronchi and noncompressed alveolar architecture were digitalized and the EPO-reactivity was quantified using a digital image analysis package (Viewfinder Lite, v1.0, 2000; Pixera Co., Burlington, MA; and Image-Pro Plus v 4.5, 2000; Media Cybernetics, Inc., Silver Spring, MD). The extent of tissue eosinophilia was expressed as EPO-positive pixels/area unit lung tissue. In separate experiments we demonstrated that the present method of EPO assessment correlates well with actual tissue numbers of eosinophils (data not shown).

Airway remodeling. **GOBLET CELL HYPERPLASIA.** Goblet cell hyperplasia and airway hypersecretion are characteristic features of allergic airways. Periodic Acid Schiff (PAS) staining of mucin granules was used to assess the number and distribution of goblet cell mucins (24). Briefly, 8 μ m cryo sections were PAS stained, rinsed in tap water, counter-stained with Methyl green and mounted (22). Goblet cells in medium-sized and large bronchi were quantified at $\times 200-400$ magnification. The length of the epithelial lining examined in each cross-sectioned bronchus (i.e., the epithelial perimeter) was calculated using digital image analysis. The degree of goblet cell hyperplasia and luminal secretions was assessed in ≥ 8 airways per animal (corresponding to $> 80-120$ analyzed airways / group) by 4 different parameters:

- 1) The proportion of airways that had goblet cell hyperplasia. Goblet cell hyperplasia was defined as airways where $> 10\%$ of the epithelial circumference contained PAS-positive goblet cells.
- 2) Mean percentage of the airway circumference with goblet cells.

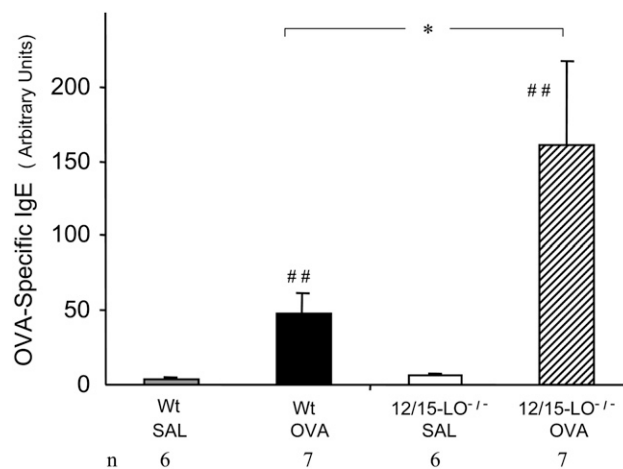


Figure 1. Augmented systemic sensitization in 12/15-lipoxygenase (LO)^{-/-} mice. Blood plasma levels of allergen (ovalbumin [OVA])-specific immunoglobulin (Ig)E were determined by ELISA in wild-type (Wt) and 12/15-LO^{-/-} mice 21 days after initial systemic sensitization (at Day 1) by intraperitoneal administration of allergen + adjuvant (control mice received saline + adjuvant). An identical booster injection was given on Day 7. Data are presented as mean values $\pm$ SEM. * $P < 0.05$, ## $P < 0.01$. The symbol # indicates differences between saline and OVA groups within the same genotype.

- 3) The extent of PAS-positive luminal secretion was assessed as the mean extent of PAS-positive secretions. This quantification of PAS-positive luminal content was performed using a semi-quantitative scale defined as 0 (absent), I (thin layer of tethered secretion lining the epithelial surface), II (small to moderate mucus plugs), III (extensive plugs covering $> 1/3^{\text{rd}}$ of the luminal surface).
- 4) The proportion of airways with any detectable PAS-positive luminal content.

EPITHELIAL CELL PROLIFERATION. Previous data have pointed out an increased epithelial cell proliferation as a sensitive index of ongoing allergic inflammation (26). Proliferating epithelial cells were assessed in cryo sections after immunohistochemical staining of the proliferation antigen Ki-67. Four similarly-sized large bronchi from each animal were selected for proliferation analysis. The numbers of Ki-67⁺ epithelial nuclei and total nuclei number were quantified along the epithelial perimeter and the mean epithelial proliferation, expressed as number Ki67⁺ nuclei/ 0.1 mm epithelial length, was calculated.

PERIPHERAL α -SMOOTH MUSCLE ACTIN-POSITIVE CELLS. Smooth muscle actin (SMA)-positive cells were assessed in paraffin sections after immunohistochemical staining of α -SMA (27). The total area of α -SMA immunoreactivity in the bronchial wall was quantified on high-resolution images of individual bronchi (27) by image analysis using a color-recognition algorithm (Image Pro Plus; Media Cybernetics). In the peripheral alveolar airways, the total number of individual SM⁺ cells per cm² lung parenchyma was quantified.

Apoptosis. In a previous study the total numbers of apoptotic cells was identified as a sensitive marker of ongoing inflammation in lung tissues (26). The present study used immunohistochemical identification of the activated form of caspase 3 (active caspase 3a) as a molecular marker of apoptosis (28). After immunohistochemical staining of caspase 3a, the total numbers of apoptotic cells were quantified in whole lung cross-sections.

Immunohistochemistry. For all immunohistochemical protocols the specificity of the staining was tested by use of control sections in which the primary antibody had been omitted. Other controls included use of irrelevant isotype-matched primary control antibodies. All controls resulted in negative staining.

α -SMA. An immunohistochemical staining using a direct-labeled monoclonal mouse anti- α -SMA (A5691, clone 1A4, Alkaline Phosphatase conjugate, 1:200; Sigma-Aldrich) was performed on 3- μ m paraffin sections (27). Briefly, sections were deparaffinized by decreasing EtOH concentrations and rinsed in TBS. The sections were incubated for 1 hour in ambient temperature with the primary antibody. The immunohistochemical reaction was developed with DAKO Fuchsin + Substrate-Chromogen system (Dako, Copenhagen, Denmark) for 6 minutes and terminated in distilled water. The slides were counterstained with hematoxylin and mounted in Kaiser's (Merck, Darmstadt, Germany) mounting medium.

CASPASE 3A. Cryosections (8 μ m) were blocked for endogenous peroxidase (0.3% H₂O₂ in methanol) in ambient temperature for 30 minutes. Blocking of unspecific binding was performed with 10% normal

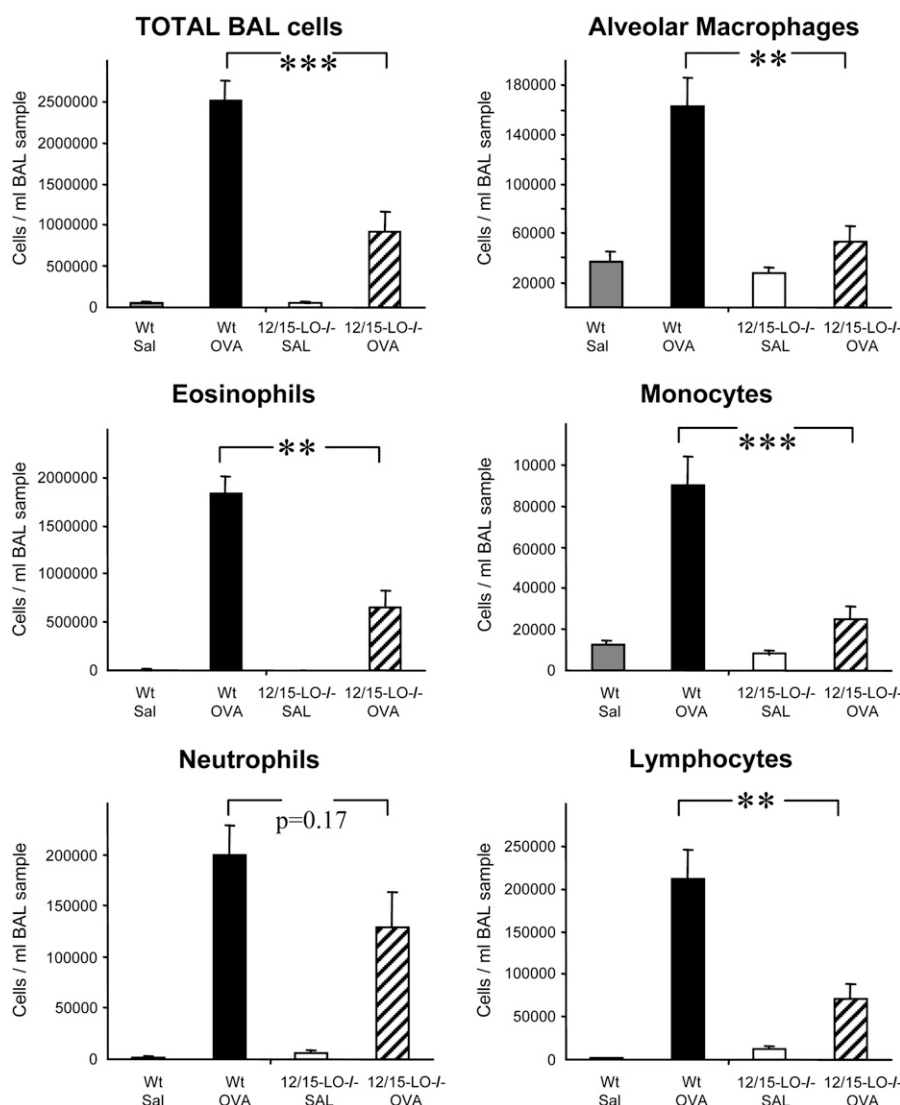


Figure 2. Attenuated luminal entry of leukocytes in airway allergen-challenged mice lacking 12/15-LO. BAL fluids were collected from single-sensitized (Day 1) animals 8 hours after four daily (Days 21–24) allergen exposures with aerosolized OVA. Data are presented as mean $\pm$ SEM of BAL levels of total and individual leukocyte cell types in saline controls or OVA-exposed animals (Wt-Sal, $n = 8$; Wt-OVA, $n = 14$; 12/15-LO^{-/-}-Sal, $n = 8$; 12/15-LO^{-/-}-OVA, $n = 10$; ** $p < 0.01$, *** $p > 0.001$).

goat serum (Vector Laboratories, Burlingame, CA) for 30 minutes. Next, the sections were incubated with primary antibody overnight at 4°C (polyclonal rabbit anti-active caspase-3, AB3623, diluted 1:100 in TBS/BSA; Chemicon International, Temecula, CA). Sections were then blocked for endogenous streptavidin and biotin with avidin/biotin-blocking kit (Vector Laboratories) and incubated for 1 hour in ambient temperature with secondary antibody (biotinylated goat anti-rabbit IgG, diluted 1:200; Vector Laboratories). Peroxidase visualization was performed during 30 minutes with Vectastain ABC kit (Elite, PK-6101, Rabbit IgG; Vector Laboratories). Detection was done with peroxidase substrate kit (DAB, SK-4100; Vector Laboratories). Next, slides were counterstained with hematoxylin and mounted in Pertex (HistoLab, Gothenburg, Sweden). All rinse steps were performed in TBS buffer.

Ki-67. Ki-67 was detected by a polyclonal rabbit anti-Ki-67 (CP 249, 1:100; Biocare Medical, Concord, CA). The immunohistochemistry protocol was as for caspase-3 above, except an additional enzymatic pretreatment of the sections before the blocking steps with 0.4% pepsin in 0.01 M HCl incubated in 37°C for 5 minutes.

Quantifications and Statistics

All quantifications were performed in a blinded manner using coded samples. The level of statistical difference between Wt-OVA and 12/15-LO-OVA (or comparisons between saline- and OVA-treated groups within each genotype) was calculated by the Mann-Whitney U nonparametric test using Astute 1.5, a statistics Add-in for Microsoft Excel (DDU Software, Leeds, UK). Values are expressed as mean $\pm$ SEM. Different *n* values between parameters is explained by a random and nonbiased exclusion of a few samples due to failure to meet the different inclusion criteria among the parameters. In the figures, the symbols * and # indicate levels of statistical differences between the groups (* or #, $P < 0.05$), (** or ##, $P < 0.01$), (***) or ###, $P < 0.001$).

RESULTS

Mice Lacking 12/15-LO Have a Fully Functioning Production of Allergen-Specific Antibodies upon Systemic Sensitization

Blood plasma titers of OVA-specific IgE antibodies were assessed in sensitized (intraperitoneal injections on Days 1 and 7) but not airway-challenged animals. Both Wt and 12/15-LO^{-/-} mice displayed significantly elevated OVA IgE levels after active sensitization compared with saline-treated animals (Figure 1). Among the OVA-sensitized animals, 12/15-LO^{-/-} mice had significantly higher levels of allergen-specific IgE (Figure 1). No significant difference was observed between the control levels of OVA-specific IgE in saline-treated Wt and 12/15-LO^{-/-} mice.

The Development of Local Airway Inflammation Is Attenuated in OVA-Challenged 12/15-LO^{-/-} Mice

Cellular inflammation. The development of an allergen-induced cellular inflammation was studied in sensitized animals after 4 days of daily allergen inhalation challenges. In BAL samples from allergen-challenged Wt and 12/15-LO^{-/-} mice, the total cell numbers, alveolar macrophages, monocytes, eosinophils, neutrophils, and lymphocytes were all significantly increased compared with saline-treated controls (Figure 2). 12/15-deficient animals had less developed cellular response compared with Wt mice, as indicated by significantly lower levels of macrophages, eosinophils, lymphocytes, ($P > 0.01$) and monocytes ($P > 0.05$). Also, the neutrophil numbers displayed a trend toward reduced levels in 12/15-LO^{-/-} mice compared with Wt animals, but for this cell type no statistically secured difference was present.

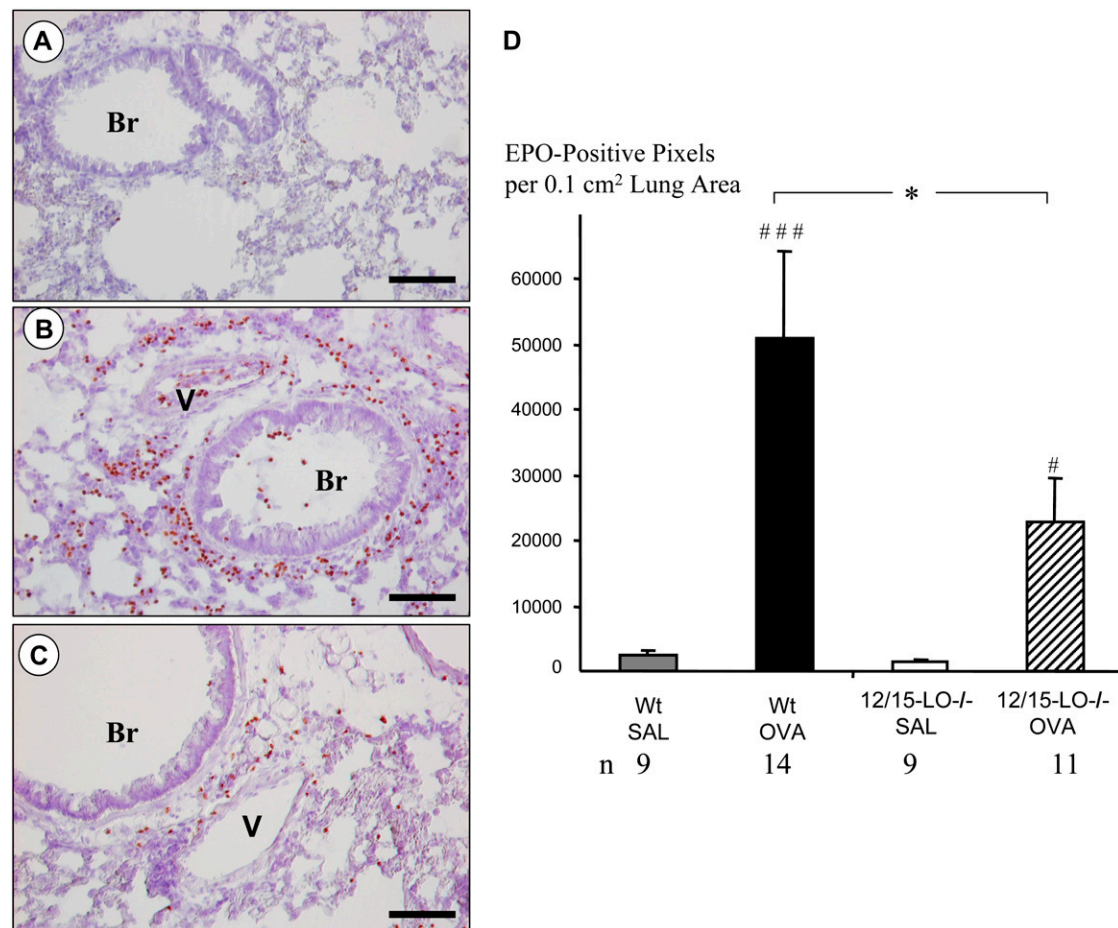


Figure 3. Reduced tissue eosinophilia in airway challenged 12/15-LO^{-/-} mice. Bright field images of eosinophil peroxidase-stained eosinophils in (A) saline-treated controls and (B) OVA-exposed Wt and (C) 12/15-LO^{-/-} mice. Lung tissues were collected 8 hours after four daily (Days 21–24) allergen exposures with aerosolized OVA. In the OVA-challenged lungs the highest eosinophil density was found in peribronchial and perivascular tissues. Br, bronchi; V, large pulmonary vessels. Scale bars = 120 μ m. D depicts mean tissue eosinophil numbers $\pm$ SEM. * $P < 0.05$, ### $P < 0.001$. The symbol # denotes differences between saline and OVA groups within the same genotype.

The cell analysis in lung tissues was focused on eosinophils, since allergic inflammation is traditionally associated with eosinophilia. There was a dramatic increase of eosinophils in allergen-challenged animals of both genotypes. The eosinophils numbers, however, were significantly lower in 12/15-LO-deficient mice compared with the Wt mice (Figure 3), but no change in the overall distribution pattern of tissue eosinophils was similar in both genotypes. The tissue eosinophilia in both genotypes had the type of peribronchial and perivascular distribution previously described in the present model (Figure 3) (22, 29), although alveolar eosinophils were also observed.

Th2 cytokines. In similarity to the BAL cell data, the levels of the Th2 cytokines IL-4, IL-5, and IL-13 in BAL supernatants were significantly increased in both genotypes after allergen challenge (Figure 4). The levels of IL-4 and IL-5, however, were statistically lower in allergen-challenged 12/15-LO^{-/-} mice compared with Wt mice. The levels of IL-13 were also reduced, although for this cytokine the *P* value was just outside the accepted level for significance (Figure 4).

Reduced Airway Remodeling in Allergen-Challenged 12/15-LO^{-/-} Mice

Epithelial alterations. The number of airways with goblet cell hyperplasia and occurrence of luminal mucus secretions were studied after PAS staining of mucins. A marked goblet cell hyperplasia developed in both Wt and 12/15-LO^{-/-} mice upon repeated allergen challenge (Figure 5). The proportion of the airways that developed goblet cell hyperplasia did not differ between the two genotypes (Figure 5G). Similarly, there was no statistical difference in the mean percentage of the airway circumference containing goblet cells between Wt ($48.3 \pm 3.6\%$, mean $\pm$ SEM) and 12/15-LO^{-/-} mice ($42.1 \pm 7.8\%$). Inspection of luminal secretions revealed, however, that Wt mice had significantly more PAS-positive luminal secretions than 12/15 LO^{-/-} mice (exemplified in Figures 5C–5F) quantified as the mean extent of PAS-positive luminal material. The mean proportion of airways containing detectable PAS-positive secretions served as another index of luminal secretion. For this parameter, both genotypes had significantly elevated levels after allergen challenge ($P > 0.01$), with the response being significantly less pronounced in 12/15 LO^{-/-} animals ($14.8 \pm 5.7\%$) compared with the Wt mice ($41.8 \pm 5.0\%$, $P = 0.0034$). Staining of the proliferation antigen Ki-67 was used as an index of inflammation-induced epithelial cell turnover and damage/repair. No apparent difference between Wt and 12/15-LO^{-/-} mice was observed regarding the baseline proliferation measured in saline-challenged, sensitized controls (Figure 5K). In OVA-exposed animals, the proliferation response was significantly increased in both genotypes, with this response being significantly weaker in the 12/15-LO^{-/-} mice (Figure 5K).

Bronchial smooth muscle. The area of the smooth muscle layer in bronchi was assessed after immunohistochemical staining of α -SMA. For both Wt and 12/15-LO^{-/-} mice, no significant increase in peribronchial SM could be detected in OVA-treated animals compared with saline-treated controls. In analogy, no difference in SM area was observed between OVA-treated Wt and 12/15-LO^{-/-} mice (Figure 6A).

Peripheral alveolar SM-positive cells. Careful inspection of sections stained for α -SMA in allergen-exposed animals revealed scattered solitary α -SMA-positive cells in the alveolar lung parenchyma (Figures 6D–6F). Quantification of these peripheral α -SMA cells revealed a statistically secured increase after OVA challenge for both Wt and 12/15-LO^{-/-} mice (Figure 6B). Notably, this allergen-induced SM response in the alveolar region was significantly lower in 12/15-LO^{-/-}

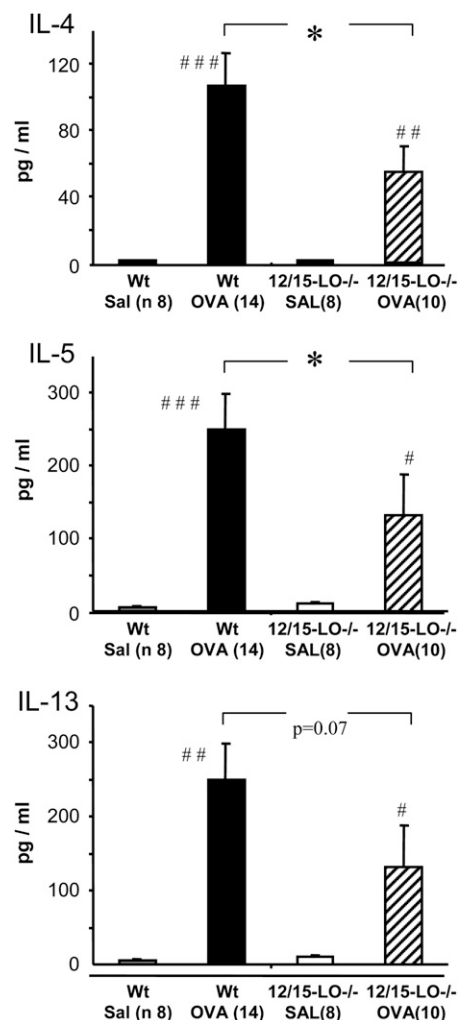


Figure 4. Attenuated Th2 cytokines in sensitized 12/15-LO^{-/-} mice after airways allergen challenge. BAL fluid levels of the Th2 cytokines IL-4, IL-5, and IL-13. BAL fluids were collected from single-sensitized (Day 1) animals 8 hours after four daily (Days 21–24) allergen exposures with aerosolized OVA. Double samples were analyzed in individual animals. Data are presented as mean of $\pm$ SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. The symbol # indicates differences between saline and OVA groups within the same genotype.

compared with similarly treated Wt mice (Figure 6B). Furthermore, there was a strong correlation between numbers of peripheral SM cells and tissue numbers of eosinophils in the same animals ($r = 0.91$, $P < 4.7 \times 10^{-17}$, Figure 6C).

Lung Content of Apoptotic Cells

The total number of apoptotic cells, which increases during an allergic airway inflammation (26), was used together with the proliferation analysis as an index of cell turnover. In the present study this parameter increased significantly after allergen challenge in both Wt ($P > 0.001$) and 12/15-LO^{-/-} mice ($P > 0.01$) compared with saline controls (Figure 7). The mean apoptosis cell number per cm² lung tissue was almost 3-fold higher in allergen-challenged Wt mice (28.8 ± 9.8) compared with 12/15-LO^{-/-} mice (10.7 ± 3.1), but no statistical difference between the two genotypes could be secured ($P = 0.11$). The major cell type undergoing apoptosis were leukocytes, and relatively few scattered structural cells showed immunoreactiv-

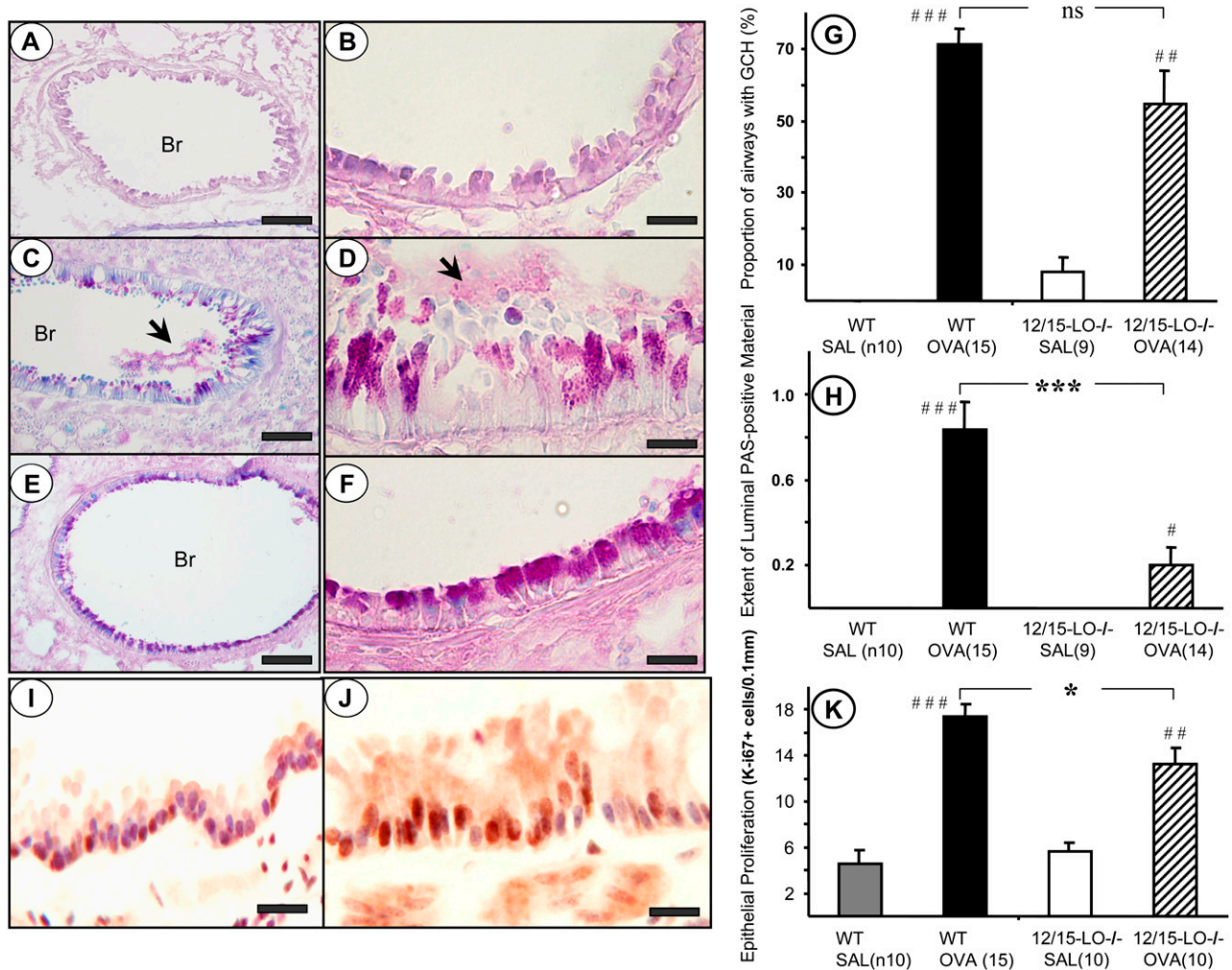


Figure 5. Sensitized and allergen-challenged 12/15-LO^{-/-} mice have fully developed goblet cell hyperplasia (GHS) but reduced hypersecretion and epithelial turnover. Low- (left) and high- (right)-power brightfield images of PAS-stained controls (A, B) and OVA-challenged Wt (C, D) and 12/15-LO^{-/-} (E, F) mice. Note the higher and goblet cell-rich epithelium in the OVA-exposed animals (C, D). PAS-positive luminal secretion is exemplified in C and D (arrows). Br, bronchi. The proportion of airways exhibiting goblet cell hyperplasia and the extent of PAS-positive luminal secretions are depicted in G and H, respectively. Panels I and J illustrate Ki-67⁺ brown cell nuclei in the epithelium from (I) a saline-treated control and (J) OVA-exposed Wt mice. Quantitative data on epithelial proliferation is shown in K. For G, H, and K, data are presented as mean of $\pm$ SEM. Numbers of animals in each group are shown in parenthesis. Scale bars: A, C, E = 120 μ m; B, D, F, I = 25 μ m; J = 20 μ m. #*P* < 0.05; ***P* < 0.01, ###*P* < 0.001. The symbol # denotes differences between saline and OVA groups within the same genotype.

ity for caspase 3a. The majority of the caspase 3a-positive cells displayed classical morphologic signs of apoptosis such as condensed cell nucleus or pyknotic cell nuclei.

DISCUSSION

The 15-LO-1 pathway has been implicated in the asthma pathogenesis. Clinical data linking 15-LO-1 to the development of allergic inflammatory airway conditions have foremost included indirect evidences such as increased expression of the 15-LO-1 enzyme or elevated BAL or sputum levels of 15-HETE²³ (12, 30). Inhaled steroids have been shown to down-regulate the expression of 15-LO-1 in patients with asthma (30). Due to the multifaceted nature of 15-LO-1-derived metabolites, which include both pro- and antiinflammatory activities, it has however been difficult to speculate about the functional consequences of the up-regulation of the 15-LO pathway in allergic disorders. Using a validated mouse OVA model, the present study explores the role of 15-LO-1 for the development and manifestation of an

airway allergic inflammation. Our data show that sensitized mice lacking 12/15-LO, the murine ortholog to the human 15-LO-1 enzyme, develop significantly less airway inflammation than do Wt mice upon local allergen challenge. This novel finding points out 12/15-LO as a primarily proinflammatory enzyme in the lung, at least during the early phases and development of an allergic inflammation. Notably, mice lacking 12/15-LO maintained a full, or even increased, capacity to produce allergen-specific anaphylactic antibodies. Hence, the present attenuated allergic airway inflammation in the 12/15-LO^{-/-} mice occurred despite a fully functioning sensitization, pointing out 12/15-LO as critical for the local manifestation of allergic airway inflammatory responses. Compared with other lipoxygenases, 5-LO-deficient mice also have a reduced airway inflammation upon sensitization and challenge with OVA (31). Airway eosinophilia induced by allergen inhalation is reduced in 5-LO-deficient mice compared with Wt mice. However, in contrast to 12/15 LO^{-/-} mice, 5-LO-deficient mice had also lower levels of serum OVA-specific IgG and total IgE.

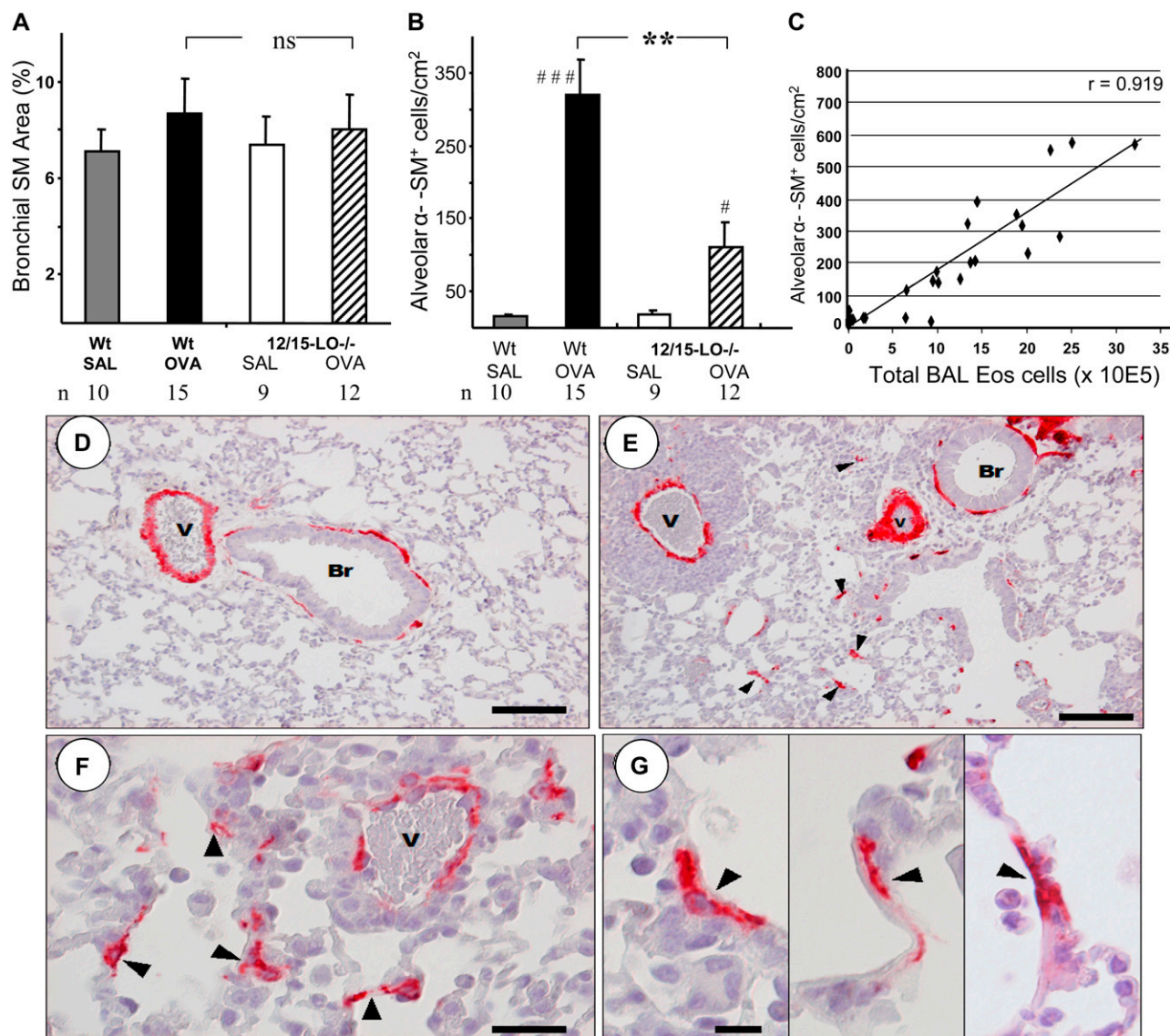


Figure 6. Attenuated numbers of free alveolar α -SMA-positive cells in allergen-challenged 12/15 LO^{-/-} mice. Lung tissues were collected 8 hours after four daily (Days 21–24) allergen exposures with aerosolized OVA. Quantitative data on (A) bronchial SM area and (B) numbers of alveolar α -SMA⁺ cells. Data are presented as mean $\pm$ SEM. A scattergram displaying the correlation between BAL eosinophils and peripheral α -SMA⁺ cells is shown in C (Spearman's Rank correlation test). Immunohistochemical identification of α -SMA after local inhalation with saline (D) or OVA-exposed Wt mice (D–F). In saline-treated controls the immunoreactivity was restricted to the smooth muscle layer surrounding vessels (V) and bronchi/bronchioles (Br). A significant feature in OVA-exposed airways is the occurrence of abundant free alveolar α -SMA-positive cells (arrowheads in E–G). High-power micrographs revealed that these cells in some cases had a truly interstitial distribution (left and middle in F) or sometimes represented cells with direct contact to the alveolar lumen (right in F). Scale bars: D, E = 80 μ m, F = 35 μ m, G = 13 μ m. * P < 0.05, ** P < 0.01, *** P < 0.001. The symbol # denotes differences between saline and OVA groups within the same genotype. The bronchial smooth muscle area (A) was quantified as percent α -SMA-immunoreactivity in the subepithelial tissue to a depth of 100 μ m.

In assessing the development of an allergic inflammation in animal models, the focus has been mainly on Th2-related cytokines and development of an airway eosinophilia. Although other significant processes are part of the *in vivo* complexity of an airway inflammation, these parameters are all elevated in human disease and have been used as main read-outs in the present type of murine model of allergic inflammation (22, 32–34). In this study a robust eosinophilia developed during the four daily allergen exposures. The magnitude of this eosinophilia and associated events like luminal entry of leukocytes and goblet cell hyperplasia was of the same magnitude as observed using prolonged 1-week allergen exposure protocols (22, 24).

The present relatively short challenge period (4 d) was not primarily designed to detect the gradual increase in bronchial SM mass that can be detected after longer challenge periods (27, 35). Indeed, the present study failed to detect any significant allergen-induced increase in total bronchial SM area. Nevertheless, several other remodeling features such as goblet cell hyperplasia and epithelial proliferation had developed, underscoring the capacity of the present model to detect early events of airway remodeling. In light of this, the present finding of markedly increased alveolar SMA cells was interesting. This observation is likely to represent a novel finding since, to the best of our knowledge, no other publication has described this phenomenon. Most of these α -SMA-positive alveolar cells had an interstitial distribution,

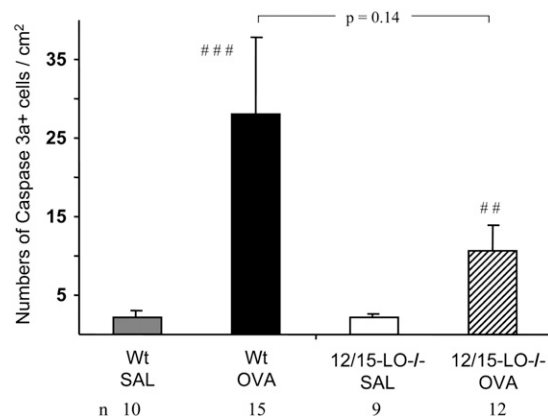


Figure 7. Numbers of Caspase 3a-positive cells in saline- and OVA-treated lungs. Caspase 3a was visualized in lung sections by immunohistochemistry. Data are presented as mean of $\pm$ SEM for each group. ### $P < 0.01$, ### $P < 0.001$. The symbol # indicates differences between saline and OVA groups within the same genotype.

suggesting that they originate from fibroblasts that during the allergic inflammation differentiate into myofibroblasts. In contrast to bronchial SM, the up-regulation of α -SMA-positive alveolar cells was markedly attenuated in 12/15-LO^{-/-} mice. Previous studies have demonstrated TGF- β to be the main factor responsible for differentiation of fibroblasts into α -SMA⁺ myofibroblasts. In both murine models of asthma and the human disease, tissue eosinophils are a major source of TGF- β and linked with airway remodeling. Notably, in the present study we could observe a surprisingly strong correlation ($r = 0.91$, Figure 6) between BAL eosinophils (many of which may be harvested from alveolar regions) and the occurrence of α -SMA⁺ alveolar cells. Hence, we suggest that the previously suggested link between eosinophils and bronchial remodeling (36–38) may also extend to peripheral parenchymal remodeling. Furthermore, our data suggest an important role of the 15-LO-1 pathway for these events.

Despite having a marked reduction in leukocyte recruitment into the lungs, 12/15-LO^{-/-} mice displayed a fully developed goblet cell hyperplasia, as assessed by numbers of goblet cells. Yet, the inflammatory mediators triggering hypersecretion did apparently not reach the threshold to cause a hypersecretory response, since the presence of luminal secretions was severely attenuated in 12/15-LO^{-/-} mice. This finding agrees with previous reports of 15-LO-1 as important, not only in expression of certain mucins like MUC8 (39), but also for active secretion. Indeed, 15-LO-1 products like 12-HETE and 15-HETE are airway mucin secretagogues (40). Lack of active secretion in 12/15-LO^{-/-} mice may also be mediated by an absence of other putative metabolites formed via the 15-LO-1 pathway. It cannot be excluded that more indirect mechanisms, like a general down-regulation of other leukocyte-derived secretagogues, are responsible for the present diminished secretory response in 12/15-LO^{-/-} mice. Likewise, it may also be speculated that a general down-regulation of local proinflammatory and mitotic factors in allergen-challenged 12/15-LO^{-/-} mice resulted in the lower proliferation rate observed in this study. Apart from reduced proliferation, the total numbers of apoptotic lung cells were decreased in 12/15-LO^{-/-} mice, suggesting an overall reduced cell turnover and intensity of the allergic inflammation.

The molecular mechanisms behind the present down-regulated allergic inflammation and associated tissue remodeling are at present unclear. The 15-LO-1 pathway and its products, such as 15(S)-HETE, 5-oxo-15-ETE, 13-hydroxyoctadecanoic acid (13-HODE), and 12(S)-HETE, have been implicated in several

key inflammatory features like leukocyte-vascular adherence, chemotaxis, migration, angiogenesis, vascular permeability, and activation of leukocytes (5, 7). However, these effects are usually observed at micromolar concentrations, which raise the question of whether these amounts can indeed be produced *in vivo*. The animal and human enzyme exhibit similar properties in many respects, but also exhibit some prominent differences. For example, the catalytic activity is to some extent different in the two enzymes. Both human 15-LO-1 and animal 12/15-LO catalyze the conversion of arachidonic acid to both 15-HETE and 12-HETE. The human 15-LO-1 possesses primarily 15-lipoxygenating activity, whereas the animal enzyme possesses primarily 12-lipoxygenating activity, and thus the spectrum of lipid metabolites could be quite different (5, 7, 41). However, eosinophils from mice have been found to produce 8,15-DHETE, 14,15-DHETE, and 14,15-leukotriene A₄ (42). The formation of these metabolites is dependent on the intrinsic 15-LO activity of the murine 12/15-LO. Thus, it is possible that the observed airway inflammation in Wt mice, but not in 12/15-LO^{-/-} mice, might at least partly be mediated by the proinflammatory eoxins, which were recently identified in human eosinophils and mast cells (17).

In summary, the present study shows that 12/15-LO^{-/-} mice, despite having an augmented systemic sensitization, had much less allergic airway inflammation and associated manifestations of central and peripheral airway remodeling. These data suggest that metabolites formed via the 12/15-LO pathway are important in the execution and establishment of local airway allergen-induced inflammation and remodeling. From these findings, it is surmised that inhibitors of 15-LO-1 would have a beneficial therapeutic effect in allergic airway diseases like asthma, allergic rhinitis, and chronic obstructive pulmonary diseases. Future clinical trials, using specific inhibitors of the 15-LO-1 pathway, are now warranted to critically test this hypothesis in patients.

Conflict of Interest Statement: H.E.C. is employed by both Karolinska Institute and Biolipox AB, a pharmaceutical company, and also has shares and options in Biolipox AB. S.S. has been employed by Biolipox since 2004. A.H. was a full time employee at Biolipox AB during the time of this study. C.K.A., K.R.T., and J.S.E. do not have any financial relationships with a commercial entity that has an interest in the subject of this manuscript.

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