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12/15-lipoxygenase expressed in non-epithelial cells causes airway epithelial injury in asthma

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The mechanisms underlying asthmatic airway epithelial injury are not clear. 12/15-lipoxygenase (an ortholog of human 15-LOX-1), which is induced by IL-13, is associated with mitochondrial degradation in reticulocytes at physiological conditions. In this study, we showed that 12/15-LOX expressed in nonepithelial cells caused epithelial injury in asthma pathogenesis. While 12/15-LOX overexpression or IL-13 administration to naïve mice showed airway epithelial injury, 12/15-LOX knockout/knockdown in allergic mice reduced airway epithelial injury. The constitutive expression of 15-LOX-1 in bronchial epithelia of normal human lungs further indicated that epithelial 15-LOX-1 may not cause epithelial injury. 12/15-LOX expression is increased in various inflammatory cells in allergic mice. Though non-epithelial cells such as macrophages or fibroblasts released 12/15-LOX metabolites upon IL-13 induction, bronchial epithelia didn't release. Further 12-S-HETE, arachidonic acid metabolite of 12/15-LOX leads to epithelial injury. These findings suggested 12/15-LOX expressed in non-epithelial cells such as macrophages and fibroblasts leads to bronchial epithelial injury.

Current estimates have shown a steep increase in the incidence of various respiratory diseases such as asthma, chronic obstructive pulmonary disease, etc[1]. So there is a necessity to explore the novel signaling pathways or novel pathogenetic mechanisms for existing candidate molecules for a better understanding of the disease and effective management. Asthma, a chronic airway inflammatory disease, is characterized by airway hyperresponsiveness to nonspecific stimuli, airway obstruction, bronchovascular inflammation, airway eosinophilia, and airway remodeling changes. These asthma features are predominantly due to imbalance in Th1/Th2 response[2,3] and this imbalance could lead to increase the proinflammatory mediators and oxidative stress in the airway which affects structural cells as a bystander effect. Among injured structural cells, airway epithelium is having crucial importance as it is the primary barrier against exogenous and endogenous stress with its innate mechanisms. Recent literature demonstrated that airway epithelia can determine the immune status of lung by recruiting particular type of immune cells through its armamentarium of cytokines such as interlekin-25 (IL-25), IL-33 and thymic stromal lymphopoietin[4–6]. Due to these reasons current respiratory researchers have given a central role for airway epithelia in pathogenesis of lung diseases[7–9]. In this context, we and others have demonstrated the importance of mitochondrial function in relation to asthmatic epithelial injury[10–12]. Restoration of mitochondrial function of alveolar epithelia can heal the lung injury indicates the enormous potency of mitochondria in maintaining epithelial health[13]. Thus, it is equally important to explore the mechanisms for the airway epithelial injury.

IL-13 is the dominant cytokine in asthma pathogenesis[14]. Its role in airway inflammation and airway remodeling including goblet cell metaplasia is well studied[15]. Though IL-13 is known to cause intestinal epithelial apoptosis[16], however it's effect in causing airway epithelial apoptosis in *in vivo* is not clear. Since IL-13 induced goblet cell metaplasia needs epithelial survival before conversion of Clara cell to goblet cells[15], the effect of IL-13 on epithelial apoptosis might have not been considered. 12/15-lipoxygenase (12/15-LOX) has been found to be consistently upregulated in IL-13 transgenic mice, ovalbumin (OVA) induced mice and also in asthmatic

bronchial epithelia[17]. On the one hand, 12/15-LOX degrades mitochondria of reticulocytes in the process of RBC maturation by peroxidizing the mitochondrial membranes[18] and on the other hand its deficiency alleviated asthma features[19]. Though our earlier studies[20,21] indicated that 12/15-LOX is linked to mitochondrial dysfunction by using vitamin E and esculetin, the direct evidence of 12/15-LOX to cause airway epithelial injury and cellular involvement are still lacking. With this context, we hypothesized that 12/15-LOX may cause airway injury in asthma pathogenesis. In this study, we have shown that 12/15-LOX present in non-epithelial cells such as macrophages leads to bronchial epithelial injury.

Results

12/15-LOX overexpression in naïve mice shows airway epithelial injury. To determine the effect of 12/15-LOX on airway injury, control or 12/15-LOX overexpression plasmid was administered intravenously or intranasally to healthy Balb/c mouse as shown in Figure 1A. Intravenous administration showed a significant expression of 12/15-LOX in lung though intranasal administration also showed a sufficient expression (Fig. 1B); so intravenous route was selected for further studies. 12/15-LOX overexpressed lungs also showed an increase in the activity of 12/15-LOX with an increase in the major metabolites such as 12-(S)-Hydroxyeicosatetraenoic acid (12-S-HETE) and 13-(S)-hydroxyoctadecadienoic acid (13-S-HODE) (Fig. 1C, D). Interestingly, 12/15-LOX overexpression plasmid administered mice had shown a spontaneous airway hyperresponsiveness (AHR), recruitment of inflammatory cells in the bronchovascular regions, increased inflammation score, moderate degree of subepithelial fibrosis and scarce goblet cell metaplasia compared to control plasmid administered mice even without allergen induction (Fig. 1E–I).

Next we wanted to determine the effect of 12/15-LOX overexpression (OE) on airway injury and mitochondrial dysfunction. 12/15-LOX OE mice had shown a decrease in complex IV activity and mitochondrial membrane potential in lung mitochondria and increase in cytochrome C in lung cytosol (CYTO) (Fig. 2A–C). Further, 12/15-LOX OE mice had shown an increase in caspase 3 activity, and increase in the number of apoptotic bronchial epithelia (Fig. 2D–F). Also there were mitochondrial structural changes such as loss of cristae in the bronchial epithelia of 12/15-LOX OE mice (Fig. 2G).

Genetic deletion of 12/15-LOX reduces airway epithelial injury. To confirm the effect of 12/15-LOX on airway epithelial injury, OVA induced Balb/c mice were administered scrambled small interfering RNA (siRNA) or 12/15-LOX siRNA (Fig. S1A). Ovalbumin–sensitized and –challenged mice showed the increased 12/15-LOX expression with increase in its linoleic acid metabolite, 13-S-HODE (Fig. S1B, C). Ovalbumin– sensitization and –challenge led to the recruitment of inflammatory cells into the airway, goblet cell metaplasia and AHR (Figs. S1D, S2A, B). Asthmatic mice treated with scrambled siRNA had the features of mitochondrial dysfunction with a reduction in the activities of mitochondrial complexes I and IV, decrease in the mitochondrial membrane potential in lung mitochondria and increase in cytochrome C in lung cytosol (Fig. 3A–D). However, 12/15-LOX siRNA treated asthmatic mice had shown the increased activities of mitochondrial complexes I and IV along with an increase in mitochondrial membrane potential in lung mitochondria and decrease in cytochrome C in lung cytosol (Fig. 3A–D). While scrambled siRNA treated asthmatic mice had shown an increase in caspase 3 activity in lung cytosol, increase in apoptosis of bronchial epithelia and mitochondrial structural changes such as loss of cristae and swelling, 12/15-LOX siRNA administered mice had shown the reduction in caspase 3 activity, reduction in airway epithelial injury and restoration of mitochondrial ultrastructural changes in bronchial epithelia, along with the attenuation of asthma features (Figs. 3E–G, S2A–H).

To confirm these findings further, we have used 12/15-LOX knockout (KO) mice (C57BL/6). Sham controls of 12/15-LOX KO mice had shown a normal mitochondrial function with no increase in cytochrome C in lung cytosol along with normal architecture of lung compared to wild type Sham controls though there was a reduction in the number of lymphocytes in bronchoalveolar lavage (BAL) fluid (Figs. 3H, S2I–L). Ovalbumin– sensitization and –challenge causes increase in the levels of cytochrome C in lung cytosol along with airway inflammation, airway eosinophilia, goblet cell metaplasia, subepithelial fibrosis, and increase in airway hyperresponsiveness in wild type C57BL/6 mice (Fig. 3H and Fig. S2I–L). However, 12/15-LOX KO mice upon ovalbumin- sensitization and -challenge had shown the reduction in cytochrome C in lung cytosol with reduction in asthma features (Fig. 3H and Fig. S2I–L).

Bronchial epithelia of normal and asthmatic mice have shown no expression of 12/15-LOX. Though it is known that there are various cellular sources of 12/15-LOX in human, 12/15-LOX expressing cells in mice are not clear. To determine the cellular involvement underlying bronchial epithelial injury, we have performed immunohistochemistry (IHC) for 12/15-LOX in lung sections of asthmatic mice and normal mice. Surprisingly, we didn't find the 12/15-LOX expression in the bronchial epithelia of normal and asthmatic mice (Fig. 4A). However, there were significant expressions in various inflammatory cells present in bronchovascular regions in asthmatic mice. Higher magnifications revealed that these cells may be granulocytes such as neutrophils and eosinophils, macrophages and monocytes in asthmatic mouse. To confirm the type of inflammatory cells that express 12/15-LOX, co-immunostaining experiments were performed in single cell suspensions of lungs. Various inflammatory cells such as eosinophils (Siglec F), macrophages (CD 11b, F4/80), dendritic cells (CD 11c), T lymphocytes (CD 3), and granulocytes (Gr-1) (Fig. 4B) had shown the increased expression of 12-LOX indicate the possible crosstalk between inflammatory cells and bronchial epithelia to cause epithelial injury.

Both normal and asthmatic individuals have shown a similar expression of 15-LOX-1 in bronchial epithelia. To determine the human relevance of observed findings in mouse, first we have performed IHC in lungs of patients with asthma and healthy individuals. More interestingly, even normal human bronchial epithelia had shown a drastic expression of 15-LOX-1 though lungs of asthmatic patients had shown the expression both in the epithelia and in inflammatory cells such as macrophages (Fig. 5A, B).

12/15-LOX expressed in non-epithelial cells induces epithelial injury. The constitutive expression of 15-LOX-1 in normal human airway indicated that epithelial 12/15-LOX may not lead to epithelial injury. Since we found an increased activeness of 12/15-LOX with increase in the metabolites in 12/15-LOX overexpressed mice which showed the bronchial epithelial injury (Fig. 1C, D), we suspect that 12/15-LOX metabolites may be crucial in causing airway epithelial injury. To determine this, we measured the levels of 12-S-HETE in sera, sputum supernatants and BAL fluid supernatants of atopic asthmatics and compared with non-atopic unrelated control individuals. There was a marked increase of 12-S-HETE in biological secretions in atopic asthmatics whereas 12-S-HETE was found to be in negligible quantity in normal individuals (Fig. 6A). The similar trend was observed in the levels of 13-S-HODE (data not shown). This indicates that human bronchial epithelia may not be responsible for the 12/15-LOX metabolites which were measured in the airway.

To determine this, bronchial epithelia were induced with IL-13 and crucial metabolite, 13-S-HODE, was determined as 13-S-HODE is produced in larger concentrations at the time of mitochondrial

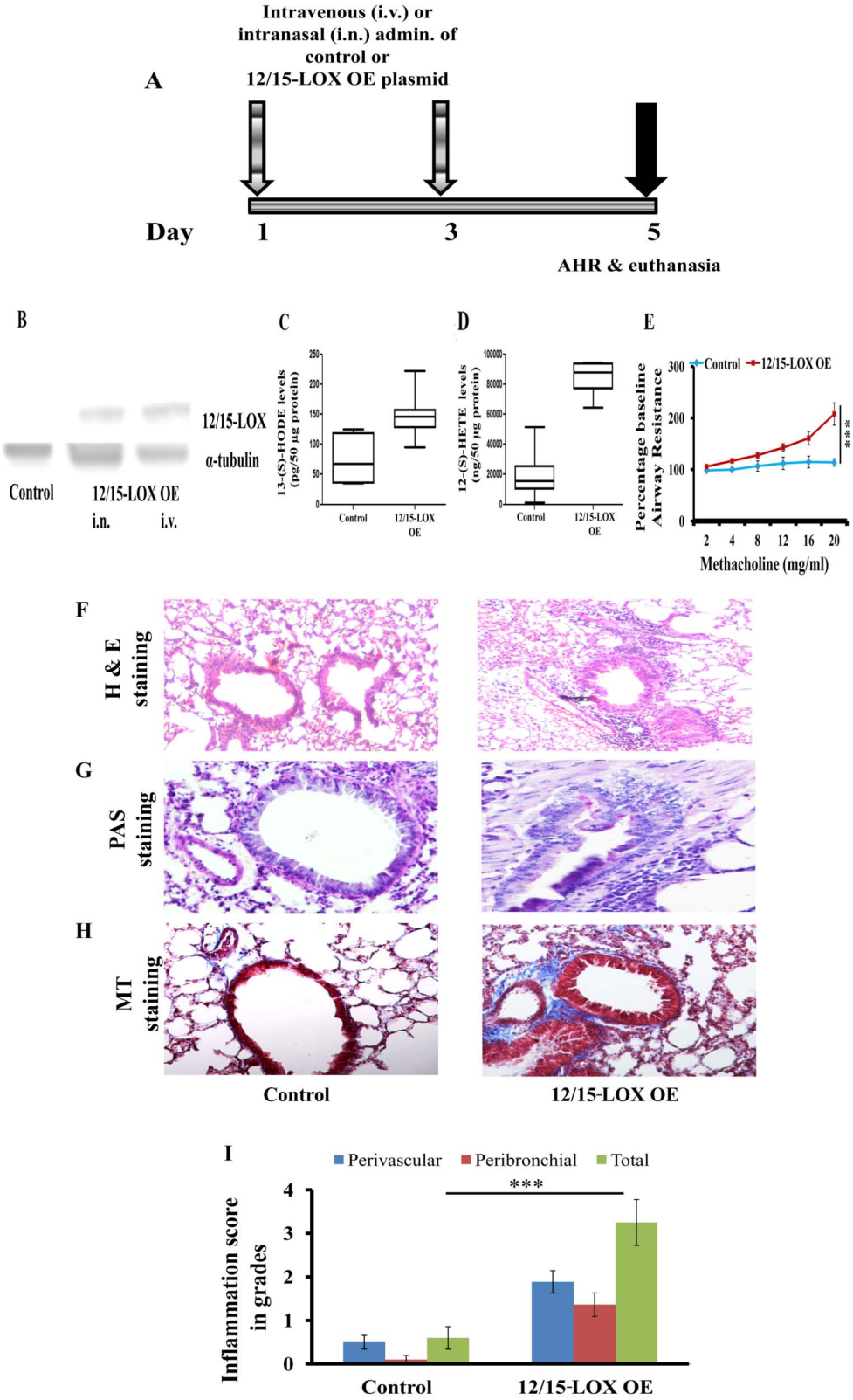

Figure 1 | 12/15-LOX overexpression in naïve mouse leads to asthma features. (A) Healthy Balb/c mice were administered either 12/15-LOX plasmid (12/15-LOX OE) or control plasmid (Control). (B–D) Western blot and ELISA to determine 12/15-LOX expression, levels of 13-S-HODE and 12-S-HETE in lung cytosol. (E) Airway resistance in response to increasing concentrations of methacholine as the percent baseline airway resistance assuming saline aerosol-derived values as baseline (n = 5–6 each group) was determined. Photomicrographs of bronchovascular regions stained with hematoxylin and eosin (F), periodic acid-Schiff (G), and Masson trichrome (H) stains of lung sections. I) Inflammation score was determined in lung sections. Data are representative of two independent experiments. Results are shown as quantile box plots for (13-S-HODE and 12-S-HETE) or as mean ± s.e.m (airway resistance and inflammation score). The line across the middle of the quantile box is the median; the ends of the box are 25th and 75th quantiles and the whiskers extending from either end of the box are the 10% and 90% quantiles. Significance is determined with unpaired Student t test or Mann-Whitney test (***$P < 0.05$).

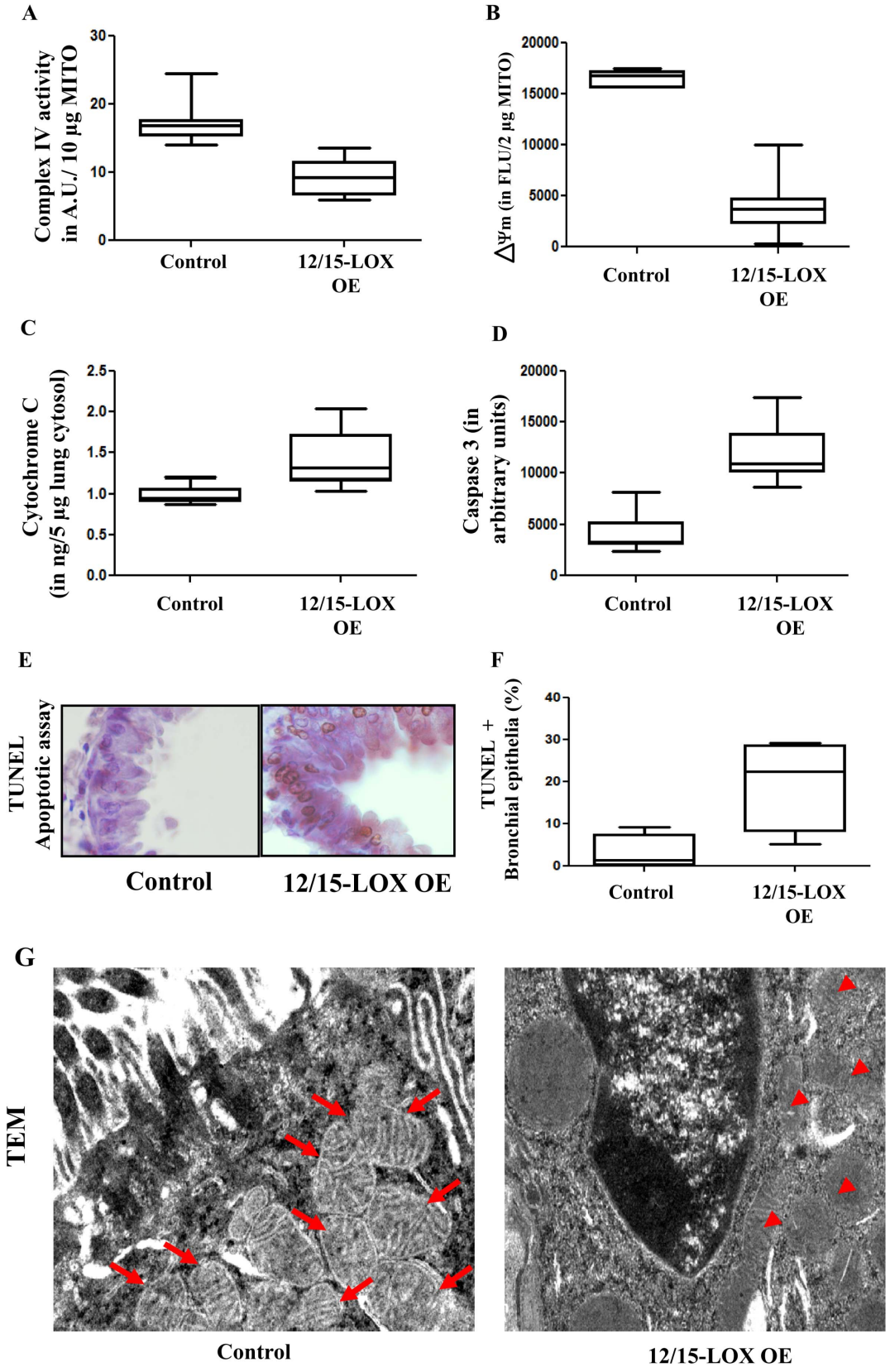

Figure 2 | 12/15-LOX overexpression in naïve mouse leads to bronchial epithelial injury. Mitochondrial complex IV activity (A), mitochondrial membrane potential (B), cytosolic cytochrome C levels (C), and cytosolic caspase 3 activity in lung cytosol (D). MITO, mitochondria. Photomicrographs of Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) stained lung sections (E) and the percentage of TUNEL-positive bronchial epithelium (F). (G) TEM images of lung sections. Red arrows indicate the normal mitochondria with well developed cristae and Red arrowheads indicate the abnormal mitochondria with loss of cristae and swelling. Results are shown as quantile box plots. The line across the middle of the quantile box is the median; the ends of the box are 25th and 75th quantiles and the whiskers extending from either end of the box are the 10% and 90% quantiles.

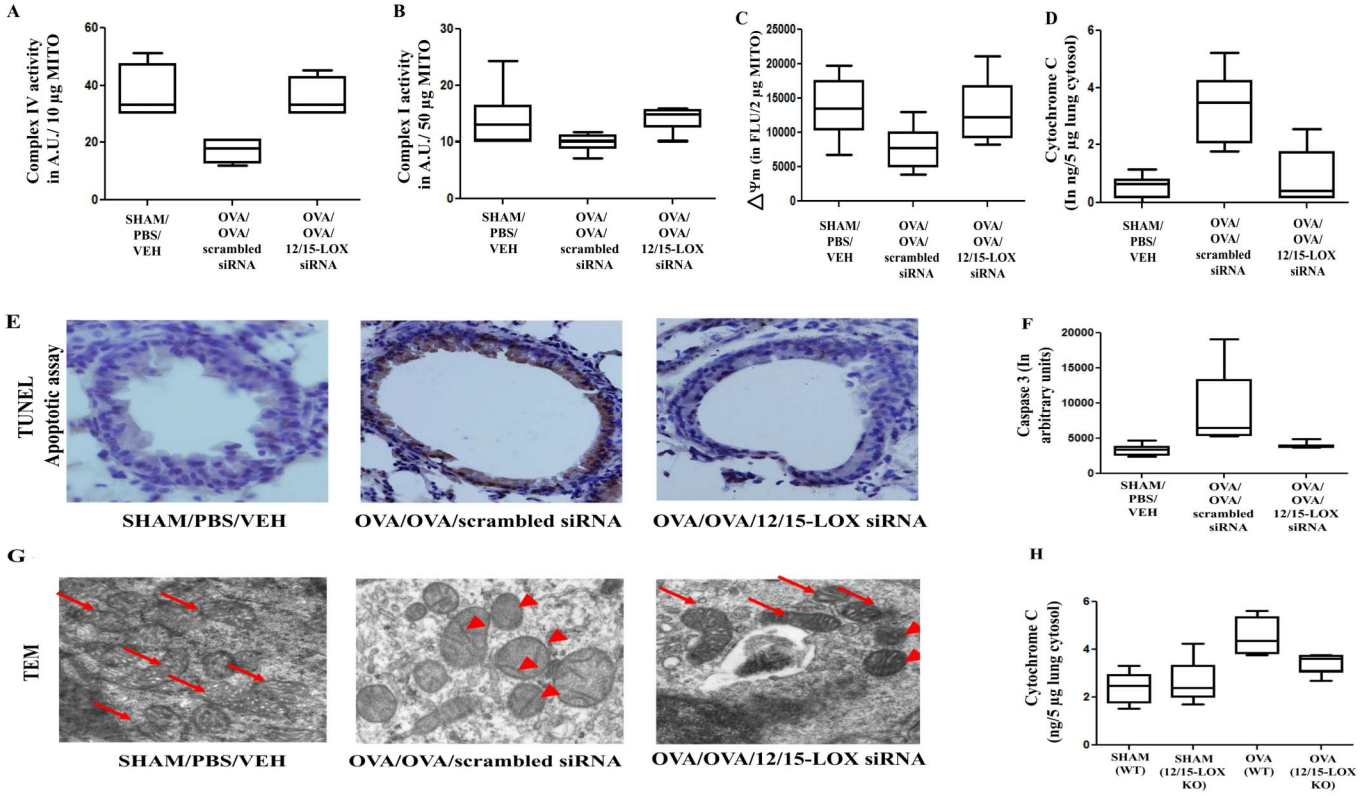

Figure 3 | 12/15-LOX deficiency reduces epithelial injury in murine asthma. Mice were grouped ($n = 5$–6 mice each group) and named as per status of sensitization/challenge/treatment. Complex IV activity (A), Complex I activity (B), Mitochondrial membrane potential (C), cytochrome C levels (D), TUNEL assay (E), caspase 3 activity of lungs (F) and TEM images of lung sections (G) in Balb/c mice. Cytochrome C levels in 12/15-LOX knockout C57BL/6 mice (H). MITO, mitochondria. CYTO, cytosol. Red arrows indicate the normal mitochondria with well developed cristae and Red arrowheads indicate the abnormal mitochondria with loss of cristae and swelling. Results are shown as quantile box plots. The line across the middle of the quantile box is the median; the ends of the box are 25th and 75th quantiles and the whiskers extending from either end of the box are the 10% and 90% quantiles.

degradation in reticulocytes[18]. IL-13 induction of human bronchial epithelial cells led to a significant increase in 13-S-HODE levels in the cytosol but not in the culture supernatant (Fig. 6B). In addition, the induction of human bronchial epithelial cells with IL-13 combination with either IL-4 or TNF-α did not increase the 13-S-HODE levels in the culture supernatant (Fig. 6C). However, IL-13 induced the release of 13-S-HODE from macrophages or fibroblasts (Fig. 6D). Further, culture supernatants of IL-13 induced macrophage reduced mitochondrial membrane potential of bronchial epithelia which was comparable to carbonyl cyanide m-chlorophenylhydrazone (cccp) treated bronchial epithelia (Fig. 6E). The mean fluorescence intensity of red aggregates (cells have healthy mitochondria) was 105.5 ± 0.5 in bronchial epithelia that were incubated with uninduced macrophage culture supernatant (mean ± sem) whereas it was 76.3 ± 0.8 in bronchial epithelia that were incubated with IL-13 induced macrophage culture supernatant. In addition, culture supernatants of IL-13 induced macrophage increase the release of cytochrome C in cytosol in bronchial epithelia (Fig. 6F). Further, exogenous 12-S-HETE, a predominant metabolite of 12/15-LOX, also released the cytochrome C in a dose dependent manner (Fig. 6F).

12/15-LOX is essential in IL-13-mediated airway epithelial injury. As we found IL-13 induced macrophage lead to epithelial injury (Fig. 6E, F) and IL-13 alone didn't lead to epithelial injury (data not shown), we wanted to determine the effect of IL-13 on epithelial injury in *in vivo* model. While IL-13 converts bronchial epithelia into mucus hyper-secreting goblet or pro-fibrotic phenotype[22], the effect of IL-13 on bronchial epithelia is not well studied. For this, we administered recombinant IL-13 (rIL-13) intranasally to Balb/c mice that were treated with scrambled small interfering RNA (siRNA) or 12/15-LOX siRNA (Fig. S3A). IL-13 administration led to increase in the 12/15-LOX expression, and in the levels of 12-S-HETE and 13-S-HODE in lung cytosol (Fig. S3B–D). Expectedly, IL-13 administration led to asthmatic features such as AHR, airway inflammation, goblet cell metaplasia, and subepithelial fibrosis (Fig. S3E, and Fig. S4A–C). Importantly, IL-13 administration leads to the features of mitochondrial dysfunction as there was a decrease in the activities of complexes I and IV and mitochondrial membrane potential in lung mitochondria and increase in the levels of cytochrome C in lung cytosol (Fig. 7A–D). In addition, we found a drastic increase in apoptotic positive bronchial epithelia in IL-13 administered mice (Fig. 7E, F). Further, IL-13 administered mice had shown the mitochondrial structural changes in bronchial epithelia (Fig. 7G). However, all these IL-13 mediated features were attenuated with 12/15-LOX knockdown (Fig. 7A–G, Fig. S3B–E, and Fig. S4A–C).

To confirm these findings, rIL-13 was administered to C57BL/6 mice those were wild type or genetically deleted for 12/15-LOX. IL-13 administered wild type mice had shown the features of mitochondrial dysfunction with decrease in the activity of complex I and increase in the levels of cytochrome C in lung cytosol (Fig. 7H–I). IL-13 induced mitochondrial dysfunction was associated with airway inflammation, goblet cell metaplasia and subepithelial fibrosis and increase in levels of 13-S-HODE (Fig. S4D–G). However, IL-13 administered 12/15-LOX KO mice had shown the restoration of the mitochondrial function along with the reduction in asthma features (Fig. 7H–I and Fig. S4D–G). These results demonstrated that 12/15-LOX is essential in IL-13 mediated epithelial injury and asthma features.

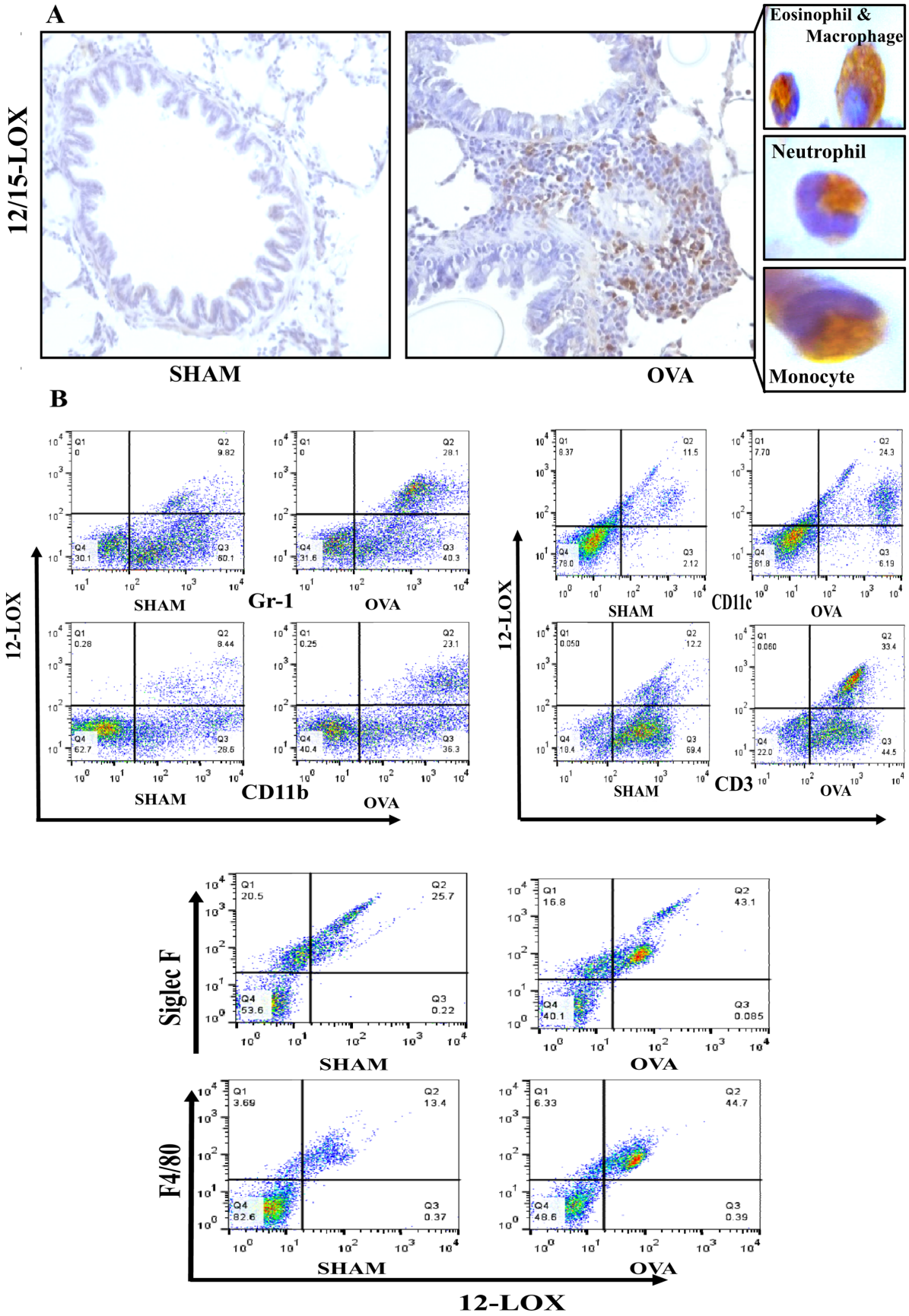

Figure 4 | Various inflammatory cells but not bronchial epithelia have shown the 12/15-LOX expression in mice. (A) The expression of 12/15-LOX in lungs of mice that are PBS sensitized and challenged (SHAM) and OVA sensitized and challenged (OVA). Brown color indicates positive expression of 12/15-LOX; Images are shown at 20× and 100× magnifications. (B) Flow cytometric co-immunostaining of 12-LOX with markers of various inflammatory cells such as Gr-1, CD11b, CD11c, CD3, Siglec F, and F4/80 in single-cell suspensions of lung tissue from SHAM and OVA mice.

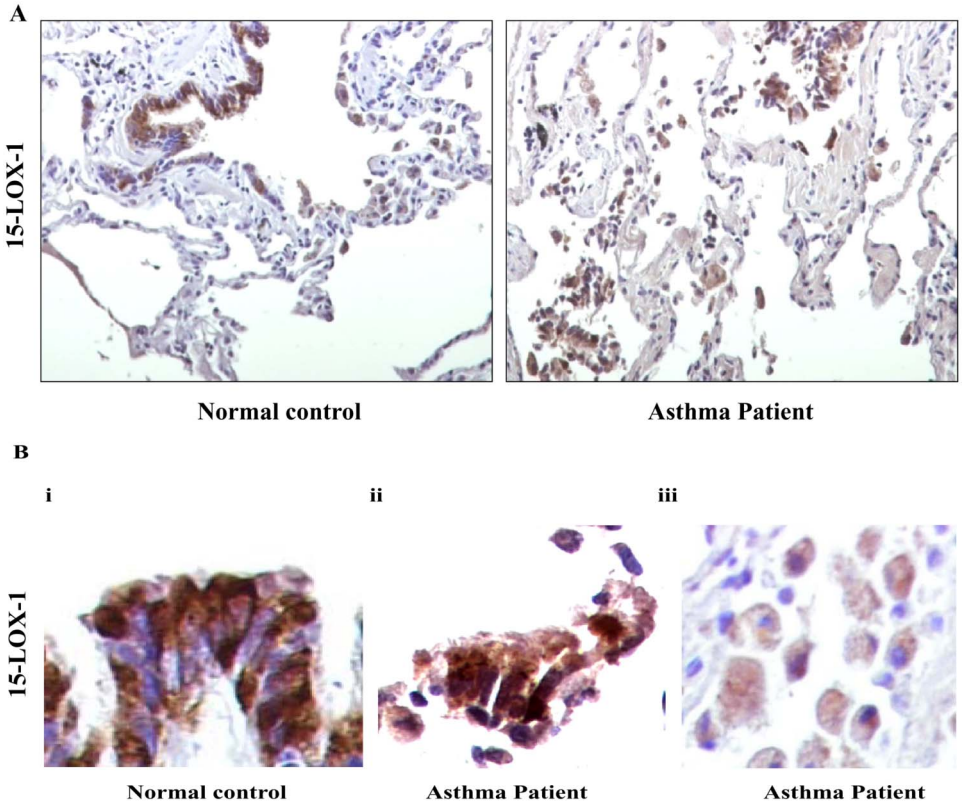

Figure 5 | Both normal and asthmatic individuals have shown a similar expression of 15-LOX-1 in bronchial epithelia. (A), (B) The expression of 15-LOX-1 in lung sections of healthy controls and patients with asthma. Brown color indicates positive expression of 15-LOX-1; all images in A are shown at 20× magnification and in B at 100× magnifications ($n = 6$ in mice and 3 to 5 in humans).

Discussion

Current human studies in asthma revealed that mechanisms for different types of asthma converge towards epithelia and suggested that epithelium could be the great contributor in asthma[23]. In this context, mitochondria seem to be crucial in airway epithelial function and pathogenesis of lung diseases[10–13]. Thus, understanding the mechanisms for mitochondrial dysfunction and airway epithelial injury are equally important for effective therapeutic strategies.

Though it is known that the deficiency of 12/15-LOX attenuates asthma features, its role in airway injury is not yet investigated. 12/15-LOX has profound biological importance since they directly oxygenates lipid-protein assemblies such as biomembranes even without prior phospholipase A2 action and it is shown to be increased in inflammatory conditions and chronic diseases such as atherosclerosis, diabetes and Alzheimer's disease[24,25]. Importantly, IL-4 and IL-13 induces 12/15-LOX expression in various cell types including bronchial epithelia[26–28]. 12/15-LOX act as an amplifier of oxidative stress as it produces fatty acyl hydroperoxides which further reacts with active redox molecules of mitochondria to generate reactive oxygen species that cause mitochondrial depolarization by forming permeability-transition pore[29]. Though this phenomenon has been explained at the physiological conditions whether abnormal increase of 12/15-LOX would have any effect on mitochondrial function and thus lung health and asthma pathogenesis was not known. In this study, we demonstrated that 12/15-LOX overexpression in naïve mice was associated with mitochondrial dysfunction and bronchial epithelial injury and on the other hand 12/15-LOX knockout/knockdown in allergic mice reduced airway epithelial injury, indicated that 12/15-LOX may cause airway epithelial injury. Importantly, 12/15-LOX overexpression also leads to spontaneous airway hyperresponsiveness even without allergen induction.

Though IL-13 is crucial in causing most of the asthma features its exact role in bronchial epithelial injury was not known. Recent success with anti–interleukin-13 therapy in asthmatic patients who had higher epithelial derived periostin levels[30] further emphasized the importance of IL-13 on epithelial function. As expected, IL-13 didn't cause epithelial apoptosis in our *in vitro* studies (data not shown), whereas it leads to significant apoptosis of bronchial epithelia in mice. These indicate the possible involvement of nonepithelial sources in causing epithelial injury. To understand this, we performed IHC for 12/15-LOX in both mouse and human asthmatic lungs. Surprisingly, there was a constitutive expression of 15-LOX-1 in bronchial epithelia of normal human beings whereas those were not expressed in murine bronchial epithelia even under asthmatic conditions. This indicated that 12/15-LOX present in other cell types could be responsible for the observed epithelial injury in 12/15-LOX overexpressed mice. Evidently, there were significant expressions in various inflammatory cells such as macrophages in asthmatic mouse and human lungs. These findings indicate that 12/15-LOX expression in epithelia may not cause mitochondrial dysfunction and airway epithelial injury and also indicated the two possible modes for epithelial injury: a) 12/15-LOX activeness and b) crosstalk between inflammatory cells and bronchial epithelia through 12/15-LOX.

Evidently, both 12/15-LOX overexpressed naïve mice and IL-13 administered naïve mice which showed mitochondrial dysfunction and airway epithelial injury also showed a significant increase in the levels of metabolites indicate the activeness of 12/15-LOX may be important for causing airway epithelial injury. Further these metabolites were absent in 12/15-LOX deficient mice which showed the reduction in airway epithelial injury. Thus, 12/15-LOX activity, production of 12/15-LOX metabolites, seems to be crucial to cause epithelial injury. To determine the activeness of 12/15-LOX in human

asthmatics, we measured 12-S-HETE in airway secretions which are close proximity to airway epithelia. Our findings showed that 12-S-HETE was found to be increased in sera, sputum and BAL fluid supernatants of atopic asthmatic patients whereas normal individuals had shown a negligible quantity of 12-S-HETE. The similar trend was observed for the levels of 13-S-HODE in our recent study[31]. These findings indicate the 12/15-LOX activeness is crucial in causing mitochondrial dysfunction.

We also found that though 13-S-HODE was found to be increased inside the cells there was no increase in the culture supernatants

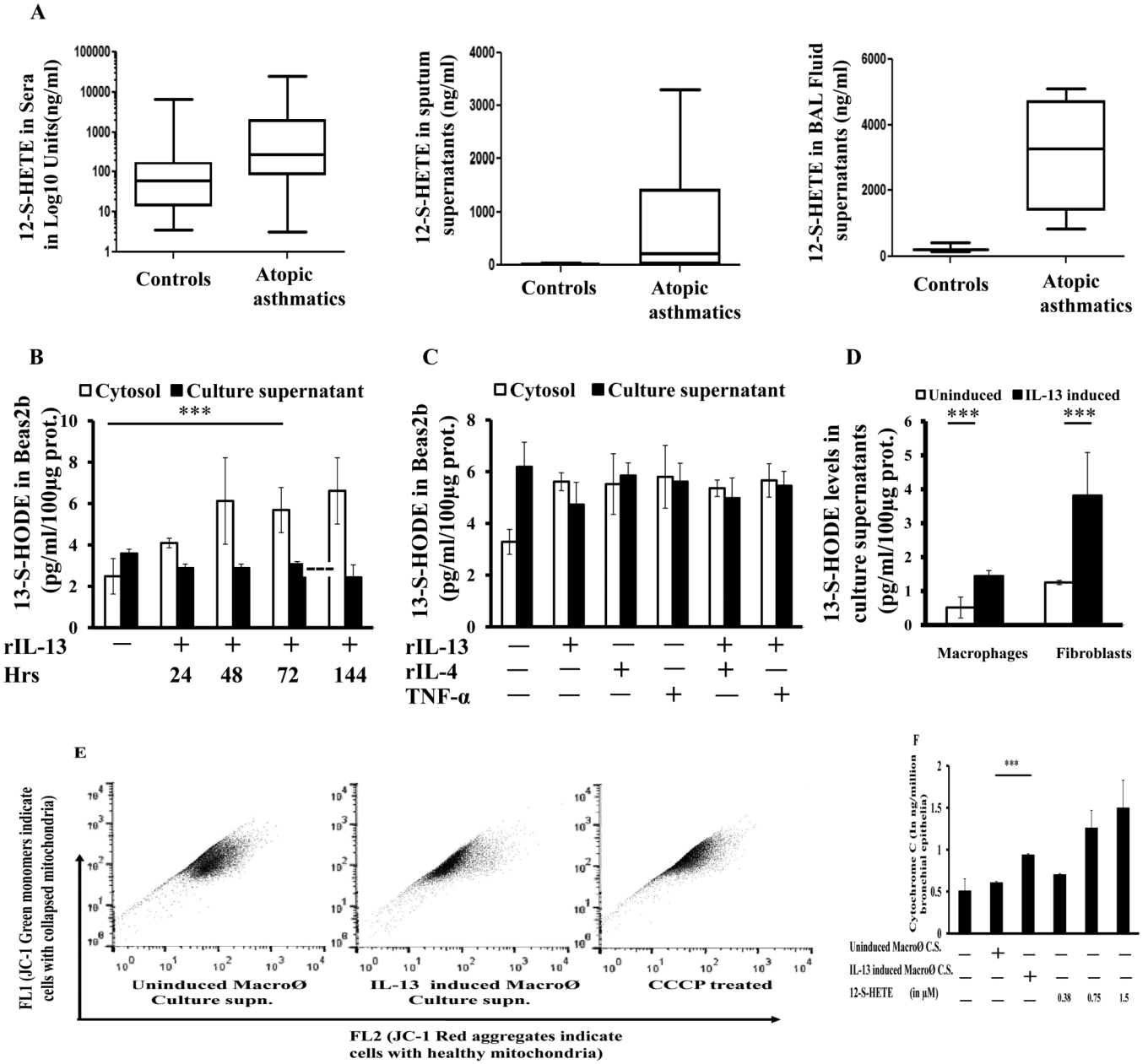

Figure 6 | 12/15-LOX metabolites induce intrinsic apoptosis in bronchial epithelia. (A) The concentration of 12-S-HETE was estimated in serum, sputum supernatant, and BAL fluid supernatant of healthy controls (Controls) or patients with atopic asthma (Atopic asthmatics). Results shown as mean ± s.e.m., significance was determined with unpaired Student's *t* test (*$P < 0.0001$). N = 40, 5, and 3 for serum, sputum, and BAL fluid supernatant, respectively, for controls and N = 80, 5, and 3 for serum, sputum, and BAL fluid supernatant, respectively, for atopic asthmatics. (B) The concentration of 13-S-HODE was estimated both in cytosol and culture supernatant of bronchial epithelium at IL-13 induction at the times indicated. (C) The concentration of 13-S-HODE was estimated both in cytosol and culture supernatant of bronchial epithelium with 48 hrs induction of either IL-13 alone or combination with IL-4 or TNF-α. (D) The concentration of 13-S-HODE was estimated both in culture supernatants of macrophages or fibroblasts at IL-13 induction for 24 hrs. Bronchial epithelia were incubated with 12/15-LOX metabolites rich culture supernatants of macrophages that were induced with 25 ng/ml IL-13 for 24 hrs and JC-1 staining (E) was performed and cytosolic cytochrome C levels (F) were estimated in those bronchial epithelia along with induction of different concentrations of 12-S-HETE. Carbonyl cyanide m-chlorophenylhydrazone (CCCP) treated bronchial epithelia stained with JC-1 was act as a positive control. MacroØ C.S., macrophage culture supernatant; Data are representative of two independent experiments. Results are shown as quantile box plots except 13-S-HODE and cytochrome C which were expressed in mean ± s.e.m. The line across the middle of the quantile box is the median; the ends of the box are 25th and 75th quantiles and the whiskers extending from either end of the box are the 10% and 90% quantiles. Significance is determined with unpaired Student t test (***$P < 0.05$).

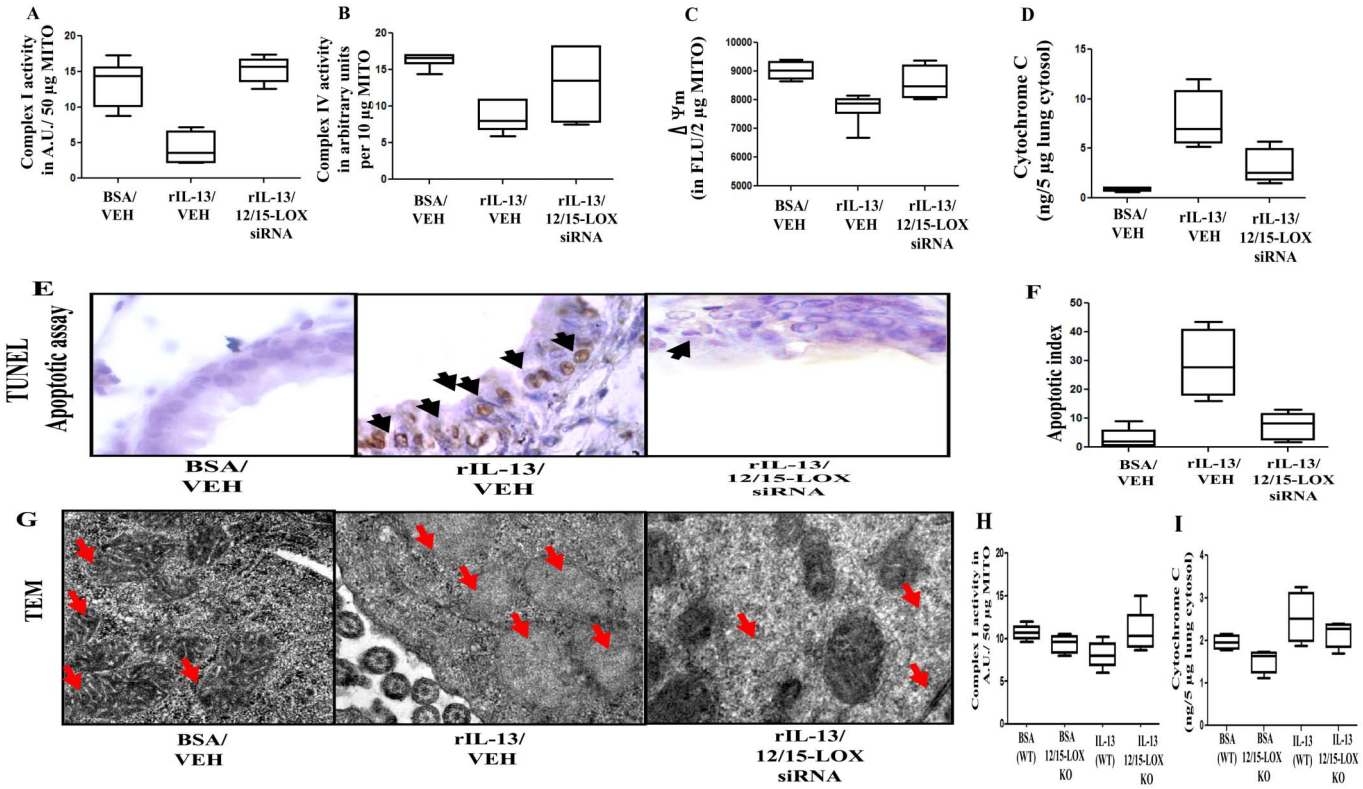

Figure 7 | IL-13-mediated epithelial injury is reduced by 12/15-LOX deficiency. Murine rIL-13 was administered as shown in Materials and Methods. Lung mitochondria and cytosols were examined for activities of complex I & IV, mitochondrial membrane potential and cytochrome C (A–D). TUNEL apoptotic assay (E) and the percentage of TUNEL-positive bronchial epithelium (F). Arrows indicated the apoptotic bronchial epithelia. (G) Ultrastructural changes of bronchial epithelial mitochondria were analyzed by TEM in Balb/c mice. Lung mitochondria and cytosols were examined for activities of complex I and cytochrome C (H–I) in 12/15-LOX Knockout mice. Results are shown as quantile box plots. The line across the middle of the quantile box is the median; the ends of the box are 25th and 75th quantiles and the whiskers extending from either end of the box are the 10% and 90% quantiles.

indicates bronchial epithelia couldn't release these metabolites. To support these findings another study demonstrated that 15-S-HETE is also not released from bronchial epithelia and intracellular 15-S-HETE leads to Muc5AC induction[32]. However, this was not true in case of other cell types such as macrophages or fibroblasts as they release 13-S-HODE. In addition, 12/15-LOX metabolites rich culture supernatants of macrophages or exogenous 12-S-HETE lead to increase the release of cytochrome C confirms that crosstalk between inflammatory cells and bronchial epithelia to cause epithelial injury. To support our findings, 12-S-HETE has been shown to increase intramitochondrial calcium overload and apoptosis in cardiac myocytes[33]. Also 13-hydroxylinoleic acid, one of the products of 12/15-LOX, had shown to increase airway hyperresponsivess in guinea pigs[34]. In this context, extracellular 13-S-HODE causes an increase in intramitochondrial calcium overload and apoptosis in bronchial epithelia[31]. These indicate that the possibly non-epithelial cells may release 12/15-LOX metabolites such as 12-S-HETE into the airway secretions and these metabolites could cause airway epithelial injury due to immediate vicinity of airway secretions to airway epithelia by activating receptors present on the epithelial surface[35–37] or diffusing in the bronchial epithelia. This needs further investigations.

The reasons and mechanisms for this differential spatial regulation are not clear right now. Collectively these indicate the existence of a delicate balance between different fates of bronchial epithelia such as goblet cell metaplasia and apoptosis. The fate of epithelia may be decided by severity of allergic inflammation and inducibility of 12/15-LOX in different cell types with IL-13. Further, 12/15-LOX has a number of other products, some of which are anti-inflammatory, such as resolvin, lipoxin, and protectin[38,39]. Thus, further studies are required to dissect the role of individual metabolites on airway injury.

In conclusion, our findings revealed a novel role in 12/15-LOX in asthma pathogenesis and also previously unknown function for IL-13 in causing 12/15-LOX mediated mitochondrial dysfunction and bronchial epithelial injury. These findings would be helpful in understanding epithelial injury and formulating therapeutic strategies targeting epithelial injury.

Methods

Animals and mice grouping. Balb/c mice (Male, 6–8 weeks, obtained from Central Drug Research Institute, Lucknow, India or National Institute of Nutrition, Hyderabad, India and maintained in IGIB, Delhi) and wild-type or 12/15-LOX knockout C57BL/6 mice (obtained from Jackson Laboratories, USA and maintained in Johns Hopkins University, Baltimore, MD, USA) were used. The respective institutional animal ethics committees approved all animal experiments. There were three different murine models and various groups as shown in Table 1.

In OVA model, mice were immunized and challenged[40,41]. Briefly, every mouse was immunized by three intraperitoneal (i.p.) administrations of 50 µg OVA (Grade V chicken egg ovalbumin, Sigma, St. Louis, MO, USA) in 4 mg alum or 4 mg alum alone on days 0, 7 and 14. Immunized mice were then challenged after a week with 3% OVA aerosol or PBS 30 min a day for 7 consecutive days with a nebulizer. Mice under isoflurane anesthesia were administered 100 µg scrambled or 12/15-LOX siRNA (Sigma, USA) intranasally. In 12/15-LOX overexpression model, pCMV6 or pCMV6-12/15-LOX was complexed with commercially available polyethylenimine (PEI) based transfection reagent (in vivo-jetPEI, Polyplus-transfection, Illkirch, France) in 5% glucose solution and injected into the tail vein (50 µg) or instilled into the nostrils (25 µg) in isoflurane anesthetized mice. Results from our earlier study[42] indicated that intravenously injected plasmids complexed with in vivo-jetPEI or in vivo-jetPEI effectively entered into the lungs. In IL-13 model, we instilled either 30 µl (3 µg) of carrier-free recombinant murine IL-13 (R&D Systems) in 1% Bovine serum albumin (BSA) or 1% BSA alone into the nasal openings in each isoflurane-anesthetized mouse.

Table 1 | Mouse models used in this study and names and details of different mouse groups

Mouse Models	Set	Groups	Details of individual group
OVA	A (Balb/c)	SHAM/PBS/VEH	Normal PBS controls
		OVA/OVA/scrambled siRNA	Allergic controls treated with scrambled siRNA
		OVA/OVA/12/15-LOX siRNA	Allergic mice treated with 12/15-LOX siRNA
	B (C57BL/6)	SHAM (WT)	Wild type normal controls
		SHAM (12/15-LOX KO)	12/15-LOX Knockout normal controls
		OVA (WT)	Wild type allergic controls
		OVA (12/15-LOX KO)	12/15-LOX KO allergic mice
12/15-LOX overexpression model	C (Balb/c)	Control	Control plasmid (pCMV6) administered mice
		12/15-LOX OE	12/15-LOX plasmid (pCMV6-12/15-LOX) administered mice
IL-13 model	D (Balb/c)	BSA/VEH	BSA administered and vehicle treated controls
		rIL-13/VEH	rIL-13 administered and scrambled siRNA treated controls
		rIL-13/12/15-LOX siRNA	rL-13 administered and 12/15-LOX siRNA treated mice
	E (C57BL/6)	BSA (WT)	Wild type bovine serum albumin administered mice
		BSA (12/15-LOX KO)	12/15-LOX Knockout BSA administered mice
		IL-13 (WT)	Wild type rIL-13 administered mice
		IL-13 (12/15-LOX KO)	12/15-LOX Knockout rIL-13 administered mice

VEH-vehicle; OVA, chicken egg ovalbumin, Grade V, Sigma.

Measurement of AHR, BAL fluid collection, histopathology and transmission electron microscopy (TEM). AHR in the form of airway resistance in anesthetized mice was determined with flexiVent system (Scireq) that uses the computer-controlled mouse ventilator to measure respiratory mechanics[40–42]. The results were expressed in the percentage baseline airway resistance with increasing concentrations of methacholine (Mch) assuming PBS aerosol induced baseline values as 100%. After the AHR measurement, bronchoalveolar lavage was performed by instilling 2 ml PBS into the tracheostomized airway and recovered BAL fluids (approximately) were processed to get cell pellet that was stained with Leishman stain to determine differential cell count as described earlier[10,40–42]. After BAL fluid collection, lungs were removed and fixed with 10% formalin. Fixed lungs were further processed and embedded with paraffin. 5-µm paraffin embedded lung sections were stained with hematoxylin and eosin, periodic acid-Schiff, and Masson Trichrome stainings for assessing the inflammation, mucus hypersecretion and subepithelial fibrosis, respectively. Stained sections were viewed and photomicrographs were taken by microscope attached with camera (Eclipse 90i). The inflammation scoring was performed by experimentally blind experts to find the perivascular, peribronchial and total airway inflammation as described earlier[10,40–42].

To determine the apoptosis in the lungs, 5-µm paraffin embedded lung sections were incubated with biotinylated nucleotides which end-labels the fragmented DNA of apoptotic cells in the presence terminal deoxynucleotidyl transferase and horse-radish peroxidase-labeled streptavidin and detected with H_2O_2, DAB system (Promega, USA). As a result apoptotic nuclei stained in brown color. Among various cell types positive for apoptosis, bronchial epithelia were the predominant cell types. So the number of apoptosis positive bronchial epithelia was determined with experimentally blind investigators to determine the apoptotic index which was expressed in the percentage positive bronchial epithelia[31,43]. To determine the ultra-structural changes in bronchial epithelia, the combined in situ perfusion and immersion fixation was performed as described previously[10]. The perfused and fixed lungs were dissected with dissection microscope (Olympus, SZX model) to find the first generation bronchi. Dissected bronchi were further processed to make blocks which were further stained and visualized with transmission electron microscope[10].

Mitochondrial experiments. Mitochondrial and cytosolic fractions were prepared from the freshly harvested lungs (Sigma, St. Louis, MO, USA)[10]. Briefly, lungs were homogenized in isotonic HEPES buffer containing fat free albumin and low speed centrifugation (600 g for 6 min) was performed to remove cellular debris and the resultant supernatants were centrifuged at high speed centrifugation (11000 g for 12 min). The resultant cell pellets were processed again with both low speed and high speed centrifugation to get mitochondrial pellets and cytosolic fractions. Both the fractions were estimated for protein quantities with BCA assay (Sigma, St. Louis, MO, USA). Mitochondrial membrane potential (Sigma, St. Louis, MO, USA), activities of mitochondrial complexes I (Mitosciences, USA) and IV (Sigma, St. Louis, MO, USA) in isolated lung mitochondria were estimated according to manufacturer's instructions[10,31].

Western blot analysis and immunohistochemistry. Lung cytosolic proteins were used for Western blot analysis of 12/15-LOX[21] (Santa Cruz, CA, USA). Briefly, lung cytosolic proteins were separated with SDS-PAGE electrophoresis, transferred to PVDF membranes those were blocked and incubated with 12/15-LOX (Santa Cruz, USA) or α-tubulin (Sigma, St. Louis, MO, USA) antibody followed by respective HRP conjugated secondary antibodies (Sigma, St. Louis, MO, USA) and the membranes were developed with DAB-H2O2 system (Sigma, St. Louis, MO, USA). Immunohistochemical analysis[31] for 12/15-LOX was performed with 12/15-LOX and respective HRP conjugated secondary antibody (Sigma, St. Louis, MO, USA). Briefly, 5-µm paraffin embedded lung sections were rehydrated, endogenous peroxidase was eliminated, incubated with 12/15-LOX antibody followed by HRP conjugated secondary antibody and developed with DAB system (Sigma, St. Louis, MO, USA). Isotype control (Jackson Immunoresearch Laboratories) or omission of primary antibody was used as a negative control.

Enzyme-linked immunosorbent assay (ELISA) and caspase 3 activity. Cytochrome C, 13-(S)-hydroxyoctadecadienoic acid (13-S-HODE), 12-(S)-Hydroxyeicosatetraenoic acid (12-S-HETE) in serum, cytosol of lung, bronchial epithelial cell lysate or culture supernatants were estimated according to manufacturer instructions. Caspase 3 activity in lung cytosols was estimated as described earlier[21].

Human participants. We have utilized sera of atopic asthmatics and healthy controls recruited for our earlier studies[44,45]. The Review Board at the CSIR-Institute of Genomics and Integrative Biology (IGIB), Delhi, India, approved the studies, and we also obtained written informed consent from the human participants. Few participants were recruited for the collection of sputum and BAL fluid with ethical approval from the Review Board at the All India Institute of Medical Sciences, New Delhi, India. The sputum induction and BAL collection were performed as described[46]. Human lung samples were obtained from Biochain (Hayward, CA, USA) and the All India Institute of Medical Sciences. Ethical Review Board at IGIB approved to use of these samples.

Cell lines. Human bronchial epithelial cells (Beas-2B), murine macrophage cells (RAW 264.7) and murine fibroblasts (3T3) were obtained (ATCC, Manassas, VA, USA). Beas-2B, RAW 264.7 and 3T3 cells were induced with vehicle, recombinant IL-13 (25 ng/ml), recombinant IL-4 (20 ng/ml) or recombinant TNF-α (20 ng/ml) (mouse or human, R&D Systems, USA). Bronchial epithelial cells were also induced with culture supernatants of IL-13 induced or uninduced macrophages or different concentrations of 12-S-HETE.

Flow cytometry. Lungs were disrupted with 70-µm cell strainer (Becton Dickinson, San Diego, CA, USA) to make single cell suspensions[41,42] which were stained with surface markers such as Gr-1, CD11b, CD11c, CD3 (BD Biosciences, USA), Siglec F (R & D systems, USA), and F4/80 (Abcam, USA) or respective isotype control antibody (BD Biosciences, USA, Abcam, USA) followed by staining with 12-LOX antibody after permeabilization. The stained cells were acquired with FACScalibur (Becton Dickinson) and analysis was performed with softwares such as Cell Quest (Becton Dickinson) and FlowJo.

To stain the bronchial epithelia with JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetra-ethylbenzimidazolylcarbocyanine iodide) dye, dual-emission potential-sensitive probe, Beas-2B cells were induced with culture supernatants of murine macrophages that were induced with 25 ng/ml IL-13 for 24 hrs and stained with JC-1 (Molecular Probes) as per manufacturer's instructions. In control experiments, Beas-2B cells were directly induced with protonopore, lipid soluble weak acid carbonyl cyanide m-chlorophenylhydrazone (cccp) which destroys mitochondrial membrane potential. JC-1 dye has dual staining properties as it forms red aggregates in a cell containing healthy mitochondria which have high membrane potential or it forms green monomers in a cell containing damaged mitochondria which have less membrane potential. The JC-1 stained cells were acquired and analyzed with FACScalibur (Becton Dickinson) equipped Cell Quest software to estimate mean florescence intensity of red aggregates (cells have healthy mitochondria).

Statistical analysis. All results except AHR and *in vitro* data are shown as quantile box plots (GraphPad Prism 5.0) which indicated median as a line across the middle of the quantile box, 25th and 75th quantiles as the ends of the box and 10% and 90%

quantiles as the whiskers extending from either end of the box. Results of AHR, *in vitro* and supplementary data are shown in mean ± s.e.m. Two-tailed Student's *t* test was used to determine the statistical significance of the differences between paired groups whereas one-way analysis of variance was used to compare multiple groups with JMP software and was further evaluated with a nonparametric Mann-Whitney rank-sum test wherever appropriate.

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Author contributions

U.M. designed, conducted, supervised, and interpreted experiments and wrote the manuscript; R.R. performed animal experiments, biochemical assays, ELISA, and qRT-PCR; T.A. performed airway physiology and imaging experiments; S.K. performed animal (C57BL/6) experiments; G.D.L. performed TEM experiments; S.S. performed *in vitro* experiments; A.K.D. supervised TEM experiments; S.B. supervised genetic knockout experiments; S.B., A.A. and B.G. provided conceptual support, interpreted experiments, and critically reviewed the manuscript; all authors reviewed the final manuscript.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: The authors declare no competing financial interests.

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12/15-lipoxygenase expressed in non-epithelial cells causes airway epithelial injury in asthma

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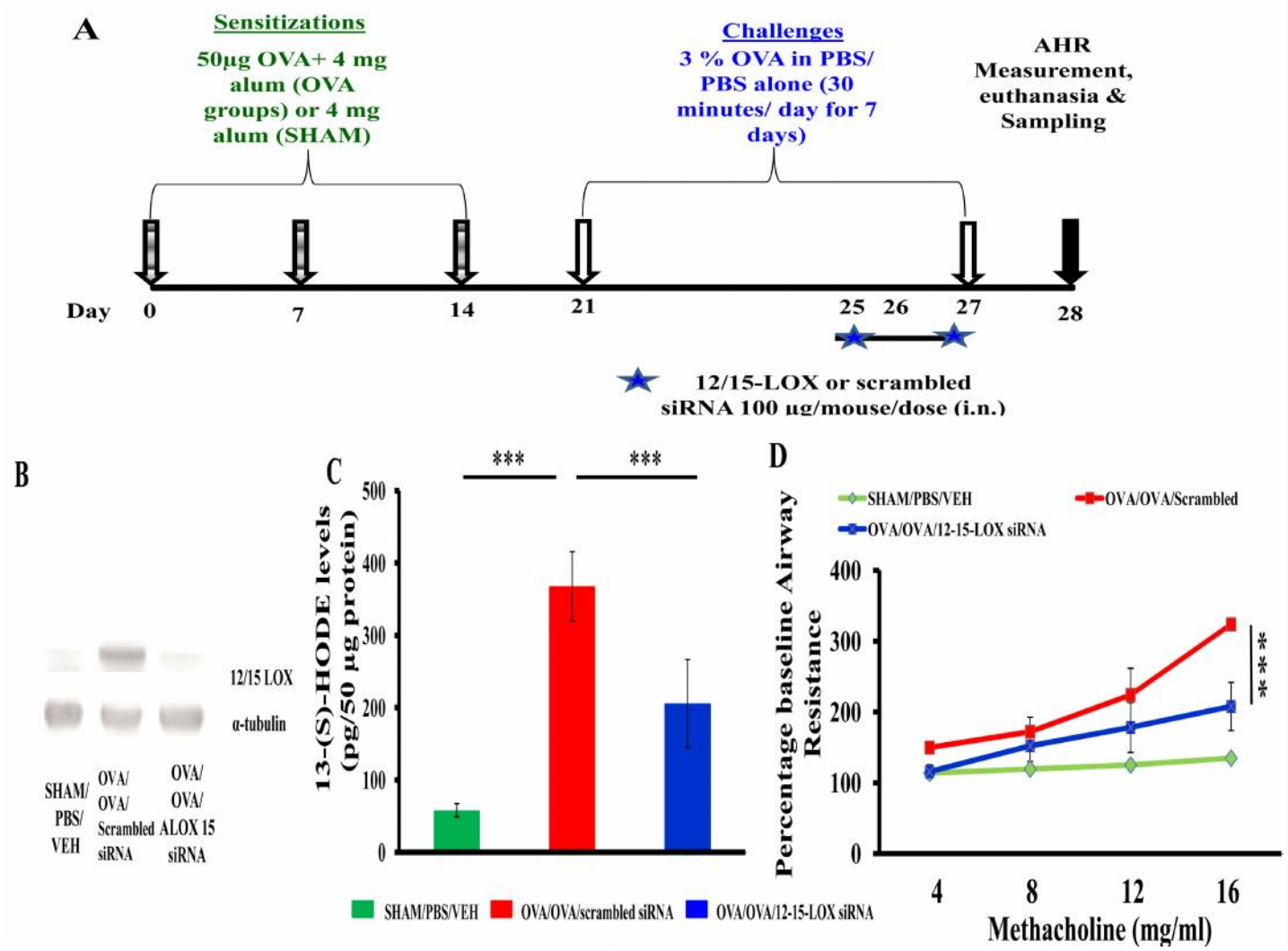
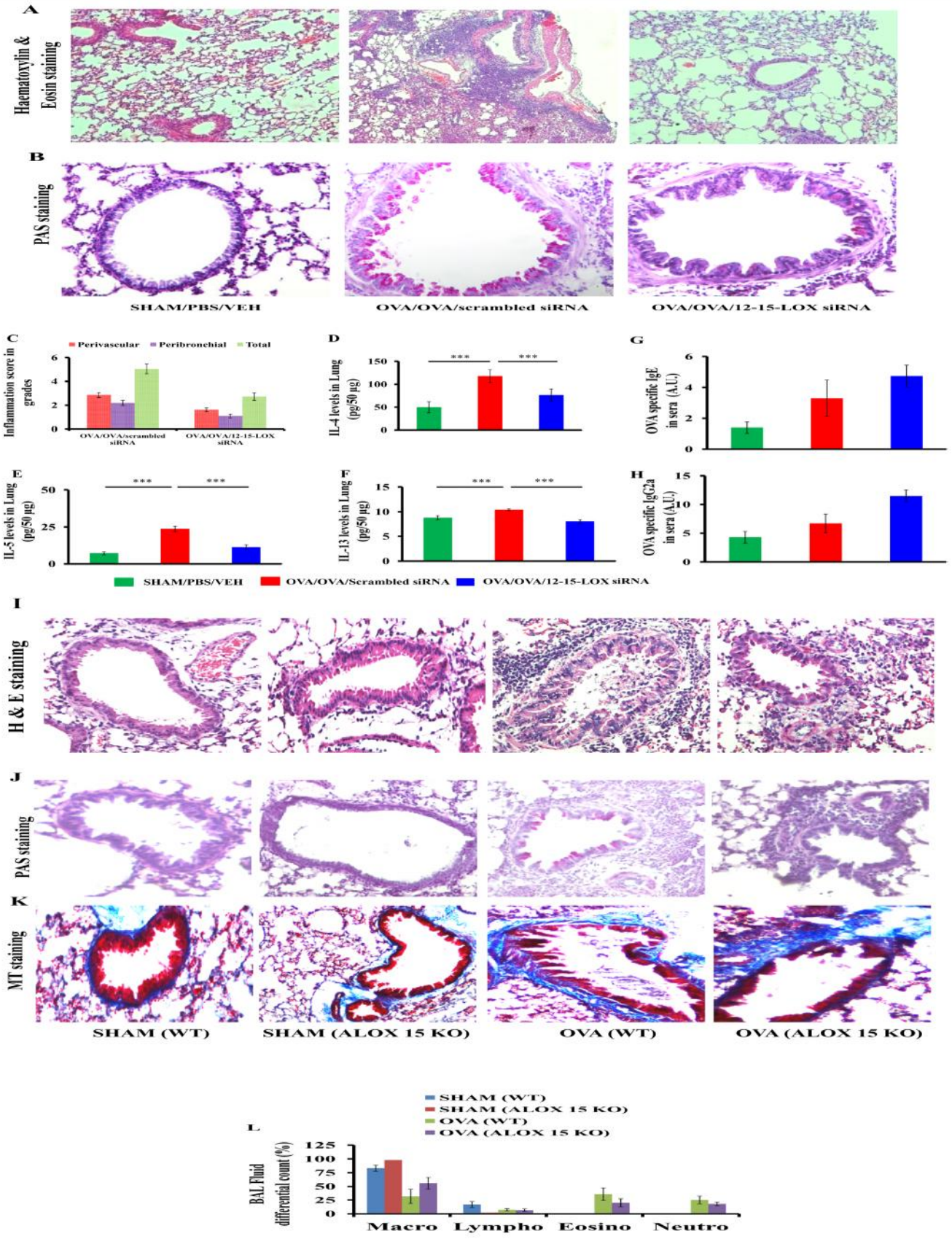
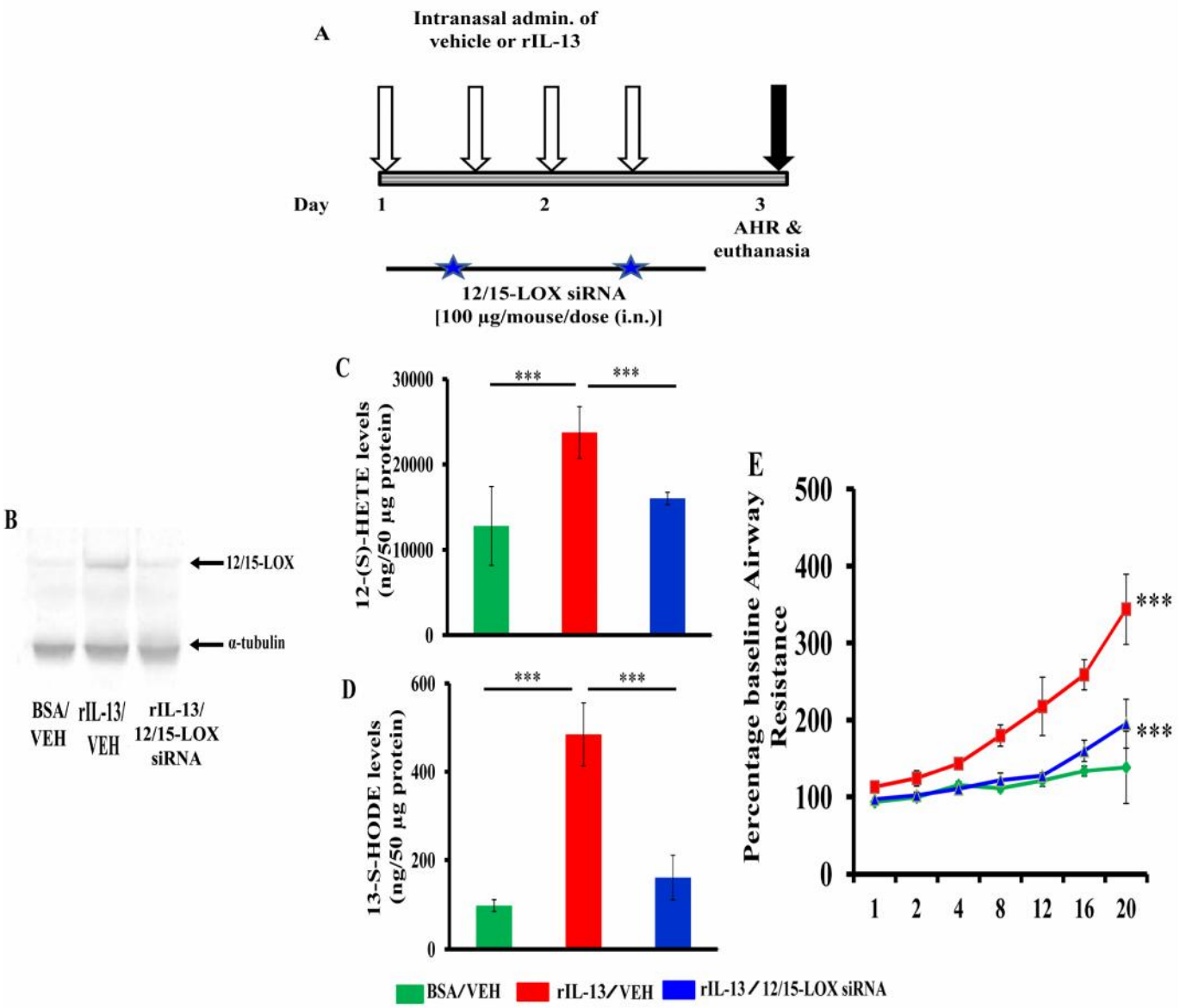


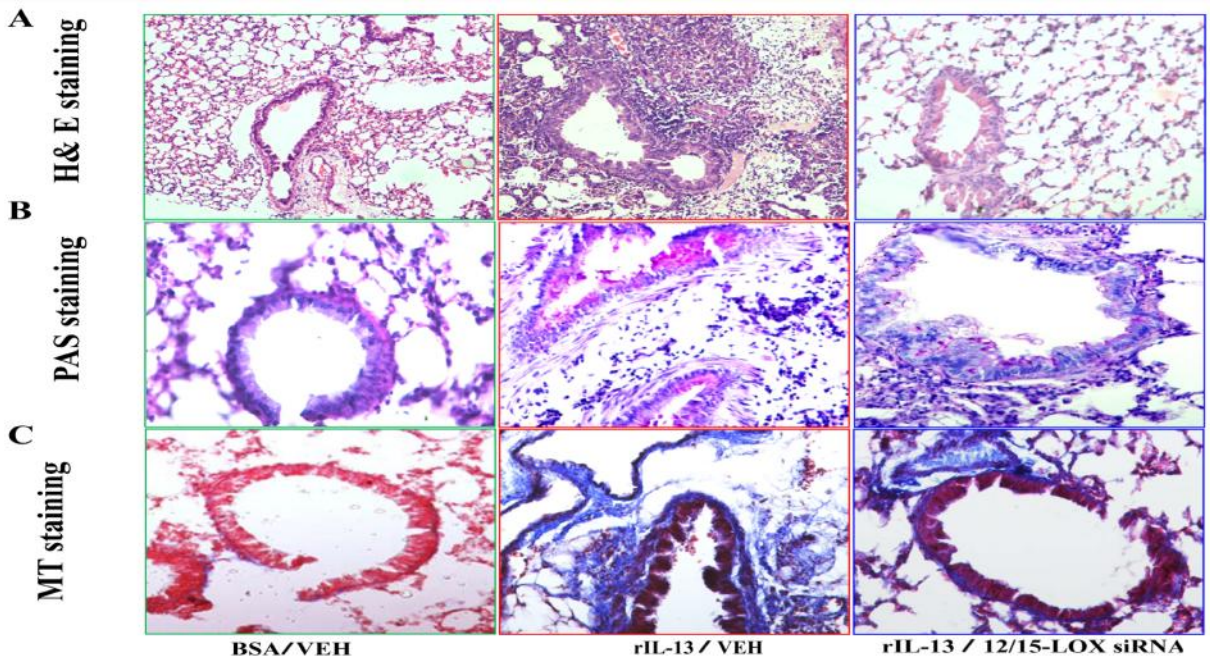
Supplementary Figure S1. Effect of 12/15-LOX deficiency on airway hyperresponsiveness in murine asthma. A) Experimental protocol to induce asthma in mouse. Mice were grouped (*n* = 5-6 mice each group) and named as per status of sensitization/challenge/treatment. SHAM/PBS/VEH, OVA/OVA/scrambled siRNA, and OVA/OVA/12-15-LOX siRNA. B , C) Western blot and ELISA to determine 12/15-LOX expression and levels of 13-S-HODE D) Airway hyperresponsiveness as percentage baseline airway resistance in response to increasing concentrations of methacholine. Results are shown as mean ± s.e.m., significance is determined with unpaired Student t test or Mann-Whitney test (***$P < 0.05$).

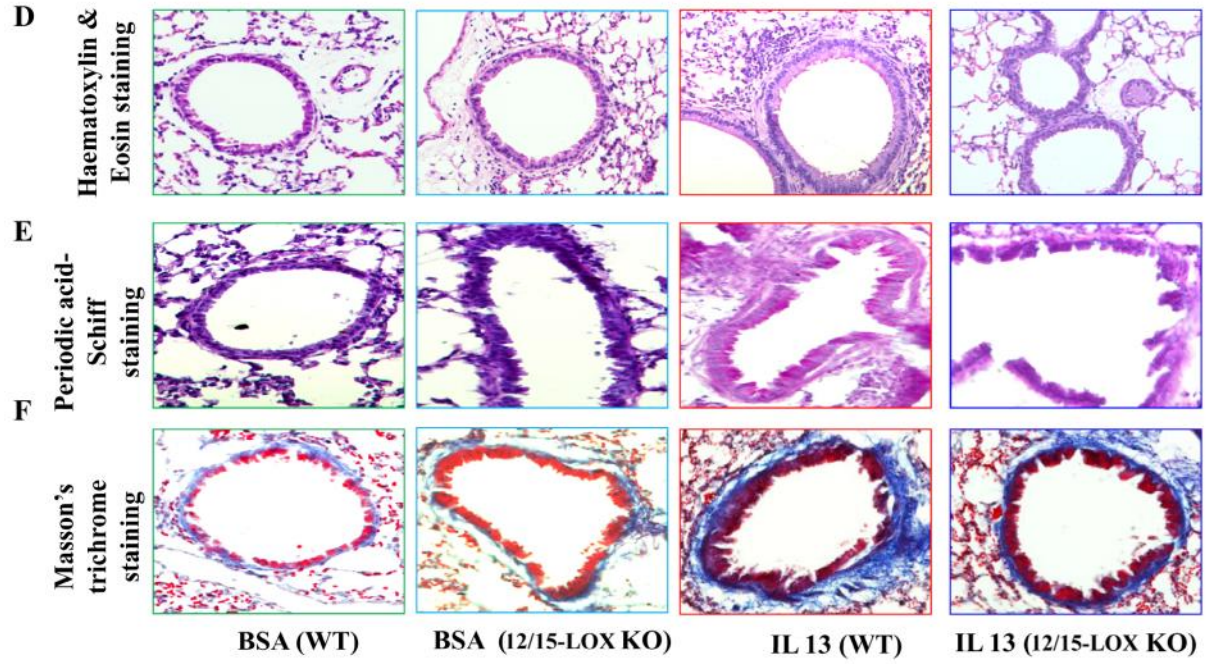

A
Haematoxylin & Eosin staining
B
PAS staining
SHAM/PBS/VEH
OVA/OVA/scrambled siRNA
OVA/OVA/12-15-LOX siRNA
C
Perivascular
Peribronchial
Total
Inflammation score in grades
OVA/OVA/scrambled siRNA
OVA/OVA/12-15-LOX siRNA
D
IL-4 levels in Lung (pg/50 µg)
G
OVA specific IgE in sera (A.U.)
E
IL-5 levels in Lung (pg/50 µg)
F
IL-13 levels in Lung (pg/50 µg)
H
OVA specific IgG2a in sera (A.U.)
SHAM/PBS/VEH
OVA/OVA/Scrambled siRNA
OVA/OVA/12-15-LOX siRNA
I
H & E staining
J
PAS staining
K
MT staining
SHAM (WT)
SHAM (ALOX 15 KO)
OVA (WT)
OVA (ALOX 15 KO)
L
SHAM (WT)
SHAM (ALOX 15 KO)
OVA (WT)
OVA (ALOX 15 KO)
BAL Fluid differential count (%)
Macro
Lympho
Eosino
Neutro

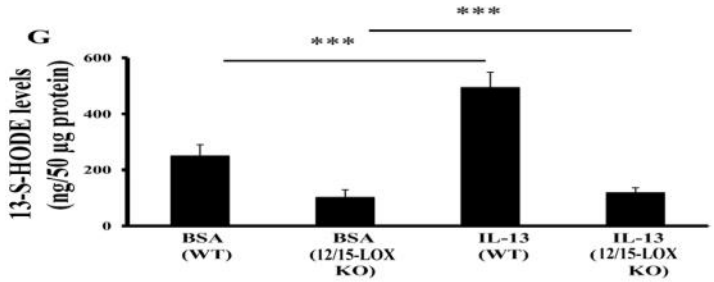
Supplementary Figure S2. 12/15-LOX deficiency attenuated asthma features in murine asthma. Representative photomicrographs of bronchovascular regions from Balb/c mice stained with H & E (A) or PAS stainings (B). Inflammation score (C), the levels of IL-4 (D), IL-5 (E), IL-13 (F), OVA specific IgE (G), and OVA specific IgG2a (H) were deteremined. Representative photomicrographs of bronchovascular regions from C57BL/6 mice stained with H & E (I), PAS (J) and MT stainings (K). L) Differential count in BAL fluid was estimated in C57BL/6 mice. Results are shown as mean ± s.e.m., significance determined with unpaired Student t test or Mann-Whitney test (***$P < 0.05$).



Supplementary Figure S3. Effect of IL-13 administration on 12/15-LOX and airway hyperresponsiveness in mouse. (A) 3-µg dose of murine rIL-13 per mouse was instilled intranasally twice a day for 2 days; 12/15-LOX siRNA (intranasal, 12 hours after the first and third rIL-13 doses) was administered in rIL-13-administered mice, and AHR was measured and euthanasia was performed 24 hours after the last dose. B) Western blot analysis of 12/15-LOX and α-tubulin in Balb/c mice. Lung cytosols were examined for the levels of 13-S-HODE (C) and 12-S-HETE (D) in lung cytosols. E) Airway hyperresponsiveness as airway resistance. Results are shown as mean ± s.e.m., significance determined with unpaired Student t test or Mann-Whitney test (***$P < 0.05$).


A
H& E staining
B
PAS staining
C
MT staining
BSA/VEH
rIL-13 / VEH
rIL-13 / 12/15-LOX siRNA


D
Haematoxylin & Eosin staining
E
Periodic acid-Schiff staining
F
Masson's trichrome staining
BSA (WT)
BSA (12/15-LOX KO)
IL 13 (WT)
IL 13 (12/15-LOX KO)


G
13-S-HODE levels (ng/50 µg protein)
600
400
200
0
***
***
BSA (WT)
BSA (12/15-LOX KO)
IL-13 (WT)
IL-13 (12/15-LOX KO)

Supplementary Figure S4. IL-13-mediated asthma features were attenuated by 12/15-LOX deficiency. Representative photomicrographs of bronchovascular regions from Balb/c mice stained with hematoxylin and eosin (A), periodic acid-Schiff (B), and Masson trichrome stains (C). Murine rIL-13 was instilled to C57BL/6 mouse that were wild type or genetically deleted for 12/15-LOX. Representative photomicrographs of bronchovascular regions from C57BL/6 mice stained with hematoxylin and eosin (D), periodic acid-Schiff (E), and Masson trichrome stains (F). 13-S-HODE levels in lung cytosols of C57BL/6 mouse. Results are shown as mean ± s.e.m., significance determined with unpaired Student t test (***$P < 0.05$).