



## Original Article

# Effect of allergy and inflammation on eicosanoid gene expression in CFTR deficiency

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## Abstract

**Background:** Allergic bronchopulmonary aspergillosis (ABPA) is a complicating factor in cystic fibrosis (CF), affecting 2–15% of patients. We hypothesized that sensitization/challenge of CFTR<sup>-/-</sup> mice with an *Aspergillus fumigatus* (*Af*) extract will affect eicosanoid pathway gene expression, impacting ABPA and CF.

**Methods:** FABP-hCFTR<sup>+/-</sup>-CFTR<sup>-/-</sup> mice were sensitized/challenged with an *Af* extract and gene expression of lung mRNA was evaluated for >40 genes, with correlative data in human CF (IB3.1) and CFTR-corrected (S9) bronchoepithelial cell lines.

**Results:** PLA2g4c, PLA2g2c, PLA2g2d and PLA2g5 were induced in response to *Af* in CFTR<sup>-/-</sup> mice. Interestingly, PLA2G2D was induced by LPS, IL-2, IL-6, IL-13, and *Af* only in CFTR-deficient human IB3.1 cells. Prostanoid gene expression was relatively constant, however, several 12/15-lipoxygenase genes were induced in response to *Af*. Numerous cytokines also caused differential expression of ALOX15 only in IB3.1 cells.

**Conclusions:** The distinct regulation of PLA2G4C, PLA2G2D and ALOX15 genes in *Aspergillus* sensitization and/or cystic fibrosis could provide new insights into diagnosis and treatment of ABPA and CF.

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**Keywords:** Allergy; Lung; Lipid mediators; Inflammation

## 1. Introduction

The eicosanoid pathway plays a defining role in inflammation, producing bioactive lipids which modulate both the onset of inflammation and its resolution. Hydrolytic cleavage of arachidonic acid (AA) from the phospholipid membrane is followed by metabolism of AA by the cyclooxygenases and lipoxygenases leading to the production of downstream prostaglandins (PGs) and leukotrienes (LTs), respectively. These pro- and anti-inflammatory lipid mediators orchestrate many aspects of inflammation including vasodilation, vascular permeability, bronchoconstriction, chemotaxis, and the transcription of pro-inflammatory enzymes; thus they are critical to several diseases such as asthma and cystic fibrosis (CF) [1–3]. Specifically in CF, the fatty acid profile is skewed

**Abbreviations:** LTs, leukotrienes; LTB<sub>4</sub>, leukotriene B<sub>4</sub>; cysLTs, cysteinyl leukotrienes; PLA<sub>2</sub>, phospholipase A<sub>2</sub>; sPLA<sub>2</sub>, secretory PLA<sub>2</sub>; cPLA<sub>2</sub>, group IV PLA<sub>2</sub>; HPETEs, hydroperoxyeicosatetraenoic acids; HETEs, hydroxyeicosatetraenoic acids; CF, cystic fibrosis; *Af*, *Aspergillus fumigatus*; CFTR, cystic fibrosis transmembrane conductance regulator; AA, arachidonic acid; COXs, cyclooxygenases; mPGES, microsomal prostaglandin E synthase; L-PGDS, lipocalin-type prostaglandin D synthase; LOX, lipoxygenase; FLAP, 5-LOX activating protein.

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toward AA, though it is not yet clear whether this is due to increased production of this precursor or decreased metabolism into the downstream products [2,3].

Within these pathophysiological events, the PGs and LTs regulate many aspects of airway inflammation and reactivity. Prostaglandins normally maintain a balance in airway responsiveness; both PGD<sub>2</sub> and thromboxane A<sub>2</sub> are bronchoconstrictors, while PGE<sub>2</sub> and prostacyclin serve in bronchoprotection [1]. On the other hand, the action of LTs appears to be principally pro-inflammatory, with LTB<sub>4</sub> possessing potent chemoattractive activity for neutrophils and eosinophils in the airways [4]. Furthermore, the cysteinyl leukotrienes (cysLTs), LTC<sub>4</sub>, LTD<sub>4</sub> and LTE<sub>4</sub>, elicit bronchoconstriction and increased endothelial membrane permeability leading to airway edema and enhanced mucosal secretion, which are also physiological hallmarks of asthma [5].

Due to this complex balance and the changes in eicosanoid metabolism that occur in CF, we hypothesized that a more detailed evaluation of eicosanoid gene expression in an animal model of asthma superimposed on CF would be valuable in predicting other therapeutic targets in eicosanoid metabolism. We developed a model of allergic bronchopulmonary aspergillosis (ABPA)/allergic asthma which employs crude *Aspergillus fumigatus* (*Af*) extract as the sensitizing and challenging agent and evaluated this in a cystic fibrosis transmembrane conductance regulator knockout (FABP-hCFTR<sup>+/-</sup>-CFTR<sup>-/-</sup>) mouse [6]. A CFTR<sup>-/-</sup> mouse model was used because asthma and *Af* sensitization are both potential complications in CF patients due to the inability to effectively clear secretions leading to infection and increased inflammation [7,8]. *Af*-sensitized/challenged mice develop a T<sub>H</sub>2 mediated allergic inflammatory response, including elevated levels of the T<sub>H</sub>2 cytokines, IL-4, IL-5 and IL-13, and increased total serum IgE, goblet cell hyperplasia and airway eosinophilia [6]. More specifically, an increase in *Af*-specific IgE and IgG is found in the serum of sensitized mice, demonstrating an *Af*-specific immune response [6]. In the present study, the gene expression levels of numerous enzymes involved in eicosanoid metabolism are analyzed by real-time RT-PCR after *Af* sensitization and challenge in CFTR<sup>-/-</sup> mice with comparisons in human CF derived and normal bronchial epithelial cell lines. Our data demonstrate alterations in gene expression for a unique subset of eicosanoid pathway enzymes which may provide relevant alternative targets in the development of markers and therapeutic regimens for CF.

## 2. Material and methods

### 2.1. Mouse strains

The original CFTR<sup>-/-</sup> mice were generated from the embryonic stem cell line, E14TGa, derived from 129/Ola mice and maintained on a C57BL/6 background [9]. Zhou et al. corrected the intestinal CFTR deficits by generating a transgenic mouse expressing the human CFTR gene (hCFTR) driven by the intestinal specific fatty acid binding protein (FABP) promoter [10]. These corrected mice, FABP-hCFTR<sup>+/-</sup>-CFTR<sup>-/-</sup> mice have a mixed background of 129/Ola, C57BL/6 and FVB/N [6,9,10]. In this manuscript, CFTR<sup>-/-</sup> refers to the mixed

background intestinally corrected mice. CFTR<sup>-/-</sup>, C57BL/6, and FVB mice were housed in the SPF facility of the University of Florida according to NIH guidelines. All experiments were approved by the IACUC of the University of Florida.

### 2.2. Sensitization and challenge

Animals were sensitized with a single lot of *A. fumigatus* extract (*Af*) (XPM3D3A4, Greer Laboratories) by intraperitoneal injections on days 0 and 14 and subsequently aerosol challenged in an acrylic chamber with nebulized *Af* extract on days 28, 29 and 30 as previously described [6]. A subset of mice was similarly sensitized and challenged with ovalbumin (OVA) while non-sensitized mice were mock-sensitized with PBS and similarly challenged with *Af*.

### 2.3. Lung mRNA extraction and real-time RT-PCR

Whole lungs from mice were flash frozen in liquid nitrogen and total RNA was isolated as per the Chomczynski and Sacchi method with modifications [11,12]. cDNA was produced with the SuperScript first-strand synthesis Kit (Invitrogen) and subsequently utilized for real-time RT-PCR with 1 × SYBR Green (Applied Biosystems, Bio-Rad). Reactions were carried out in an ABI Sequence Detection System 7000 and relative fold changes were determined using the  $\Delta\Delta CT$  method normalized to cyclophilin A. Briefly, the difference between crossing threshold (CT) values of the target and control genes represents  $\Delta CT$  value. The difference between the  $\Delta CT$  value for any given sample and the control sample generates the  $\Delta\Delta CT$  value.  $2^{-\Delta\Delta CT}$  provides the relative fold change for each sample compared to the control, which is normalized to 1. Primers used are listed in Supplemental Table 1. Human and mouse gene symbols, based on HUGO and Mouse Genome Informatics guidelines, used throughout this study are detailed in Supplemental Table 2.

### 2.4. Tissue culture

SV40 T-antigen transformed human bronchial epithelial cells from a CF patient (IB3.1) [13] and IB3.1 cells corrected through the expression of an AAV inserted functional CFTR gene (S9) [14] were maintained in Ham's F12K medium (Life Technologies) with 10% fetal bovine serum, 10 µg/mL penicillin G, 0.1 mg/mL streptomycin and 0.25 µg/mL amphotericin B at 37 °C in 5% CO<sub>2</sub>. Cells were grown to 70–75% confluency before 12 h of treatment with pharmacologic agents and/or cytokines. Treatment concentrations were as follows: *Af*, 100 µg/mL; LPS, 0.5 µg/mL; IFN $\gamma$ , 5 ng/mL; TNF- $\alpha$ , 10 ng/mL; IL-1 $\beta$ , 2 ng/mL; IL-2, 5 ng/mL; IL-3, 5 ng/mL; IL-4, 20 ng/mL; IL-6 10 ng/mL; IL-10, 20 ng/mL; and IL-13, 20 ng/mL.

### 2.5. Statistical analysis

The data from each group of mice were pooled and analyzed by two-tailed unpaired Student's t-tests. Cell line studies were performed based on animal data and were analyzed by one-tailed

paired Student's t-tests. Statistical significance was determined as  $p \leq 0.05$ .

### 3. Results

#### 3.1. Allergic and inflammatory model in CF

We previously evaluated *Af* extract as an allergen in C57BL/6 and CFTR<sup>-/-</sup> mice as a model of allergic asthma demonstrating that *Af*-sensitized mice develop a Th2-mediated response as seen in asthma with increases in total serum IgE, goblet cell hyperplasia and airway eosinophilia [6]. As indicated in 2.1 Material and methods, we chose a mixed background mouse strain, FABP-hCFTR<sup>+/-</sup>-CFTR<sup>-/-</sup> [6,9,10], which may be more analogous to a genetically and immunologically diverse human population. We have performed similar analyses in two normal inbred mouse strains, C57BL/6 and FVB (summarized in Table 2), due to their contribution to the mixed background of the FABP-hCFTR<sup>+/-</sup>-CFTR<sup>-/-</sup> mice [6,9,10]. It should be noted that even if a gene responds differently in the knockout mouse compared to each of the background strains, it is impossible to conclude whether differences are due to the CFTR gene ablation or a combination of factors arising from the mix strain background. Therefore, any response due to the lack of a functional CFTR was further substantiated in human cell lines. Specifically, we have utilized bronchoepithelial cells isolated from a cystic fibrosis patient and immortalized with a hybrid virus, adeno-12-SV40 expressing SV40 T-antigen, IB3.1 [13]. As a control, we provide results from the S9 cell line, derived from the IB3.1 cell line by introduction of a wild-type CFTR gene harbored in an adeno-associated viral vector [15]. Differences between the CF cell line, IB3.1, and the corrected S9 cells have been previously highlighted electrophysiologically [16] and in the inflammatory response [17], with the consistent implication that S9 cells behave similarly to normal bronchoepithelial cells. In this way, the mixed background of the mice allows for observations that may be linked to the CFTR gene, while subsequent studies in the human cell lines allow for a direct comparison in which the only difference is the presence of a functional CFTR.

Our previous study evaluated the induction of specific cytokines and chemokines in *Af* sensitized/challenged C57BL/6 and CFTR<sup>-/-</sup> mice [6] with similar results in FVB mice (data not shown). To further demonstrate the effectiveness of *Af* in lung inflammation, we studied the induction of similar inflammatory mediators in response to *Af* compared to the more commonly used sensitizing antigen, ovalbumin (OVA). These results demonstrated a similar response with only a difference in magnitude between *Af* and OVA-treated animals (Supp. Fig. 1). As a clinically relevant antigen, this comparison with OVA further validates the use of *Af* as an experimentally relevant allergen.

#### 3.2. Gene expression of the phospholipase A<sub>2</sub>s

Given the importance of eicosanoids in lung disease [18], including CF [3], we next studied changes in lung gene expression for a large repertoire of enzymes involved in the eicosanoid pathway in *Af* sensitization/challenge in CFTR<sup>-/-</sup>

mice. This pathway (Supp. Fig. 2) was targeted due to the compounding clinical manifestations of asthma in some CF patients with linkage to *A. fumigatus* [19]. Supplemental Table 2 summarizes all the genes analyzed for lung expression providing both the mouse and human gene names and symbols with corresponding NCBI accession numbers. We first evaluated the phospholipase A<sub>2</sub> (PLA<sub>2</sub>) family, composed of several groups of secretory PLA<sub>2</sub>s and two groups of cytosolic PLA<sub>2</sub>s, groups IV (cPLA<sub>2</sub>s) and VI (iPLA<sub>2</sub>s), which is responsible for the initial release of the primary metabolite, arachidonic acid (AA). Lung gene expression was increased for three secretory phospholipases, Pla2g2c, Pla2g2d and Pla2g5, in *Af* sensitization/challenge CFTR<sup>-/-</sup> mice compared to PBS (Fig. 1A). We previously studied these genes in C57BL/6 mice, where only Pla2g2e and Pla2g5 were induced. All of these results and data from C57BL/6 [20] and FVB mice are summarized in Table 2.

Due to the significant response seen by Pla2g2d and Pla2g5 in CFTR<sup>-/-</sup> mice, we then studied the homologous genes in human IB3.1 and S9 cells. Of these two cell lines, only IB3.1 demonstrated an induction of PLA2G2D in response to a panel of stimuli (Fig. 1B). In the case of PLA2G5, we observed an induction in both cell lines only in response to TNF- $\alpha$  (data not

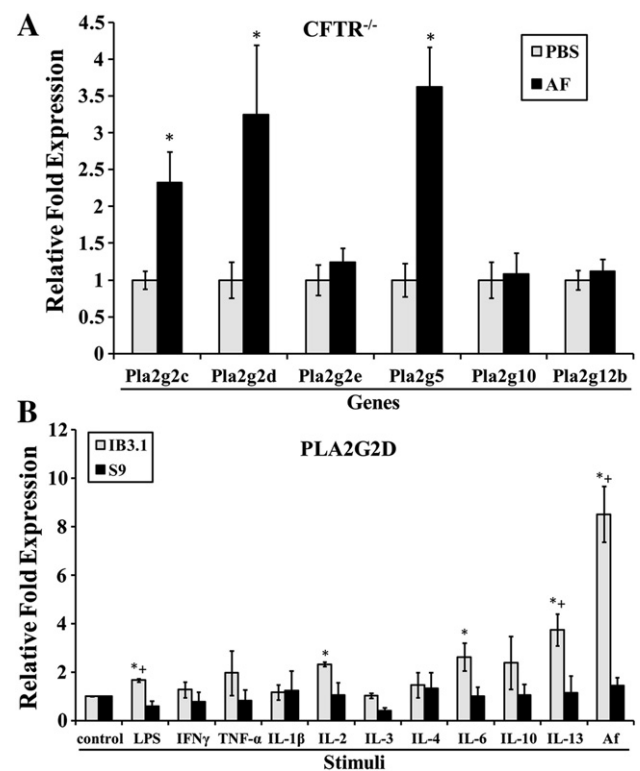


Fig. 1. Secretory phospholipase A<sub>2</sub> steady state mRNA levels in mouse lungs and human cell lines. (A) CFTR<sup>-/-</sup> mice were sensitized/challenged with PBS or *Af*. Real-time RT-PCR was performed from whole lung mRNA for groups II, V, X and XII PLA<sub>2</sub>s: Pla2g2c, Pla2g2d, Pla2g2e, Pla2g5, Pla2g10 and Pla2g12b. Data points are the means of  $2^{-\Delta\Delta CT} \pm SEM$  ( $7 \leq n \leq 10$ ). \* indicates  $p \leq 0.05$  as compared to PBS-sensitized mice. (B) Real-time RT-PCR of PLA2G2D following a 12 h treatment of S9 and IB3.1 cells with the indicated inflammatory stimuli. Data points are the means of  $2^{-\Delta\Delta CT} \pm SEM$  ( $n=3$ ). \* and + indicate  $p \leq 0.05$  as compared to IB3.1 control and treated S9 cells, respectively.

shown). Of note, we have also previously demonstrated that various cytokines, including IL-6, IL-10, IFN $\gamma$ , and TNF- $\alpha$ , are elicited from murine primary alveolar macrophages in response to the *Af* extract, providing additional relevance to these stimuli [20].

We next evaluated the expression levels of three members of the group IV cytosolic phospholipases (cPLA $_2$ ), Pla2g4a, Pla2g4b, and Pla2g4c, with only Pla2g4c expression displaying a significant increase in *Af*-sensitized/challenged CFTR $^{-/-}$  mice (Fig. 2). We observed a similar response in C57BL/6 [20] and FVB mice (Table 2), as well as an analogous induction of Pla2g4c mRNA levels by OVA-sensitization/challenge, in support of the experimental relevance of *Af* as a sensitizing agent (Supp. Fig. 3). We similarly tested the expression of PLA2G4C in IB3.1 cells which displayed substantial inductions by the pro-inflammatory stimuli TNF- $\alpha$  and IL-1 $\beta$  (Table 1), with no significant differences as compared to our results in S9 cells [20].

### 3.3. Gene expression analysis of enzymes involved in prostanoid synthesis

Eicosanoid biosynthesis following the liberation of AA from membrane phospholipids can result in the metabolism of AA by cyclooxygenases (COXs) and specific downstream synthases or lipoxygenases (LOXs). The relative expression levels of cyclooxygenases (Ptgs1 and Ptgs2), microsomal prostaglandin E synthase-1 (Ptges), thromboxane A $_2$  synthase (Tbxas1), lipocalin-type prostaglandin D synthase (Ptgds) and prostaglandin I $_2$  synthase (Ptgis) were analyzed. We observed a small upward trend in the expression of Ptgs1, Ptgs2 and Ptges in the CFTR $^{-/-}$  mice (Fig. 3). This small increase in Ptges was also observed in C57BL/6 mice (Supp. Fig. 4) and summarized in Table 2. With no statistical significance to these trends, follow-up studies were not performed in human cell lines.

### 3.4. Gene expression analysis of enzymes involved in leukotriene (LT), HPETE and HETE synthesis

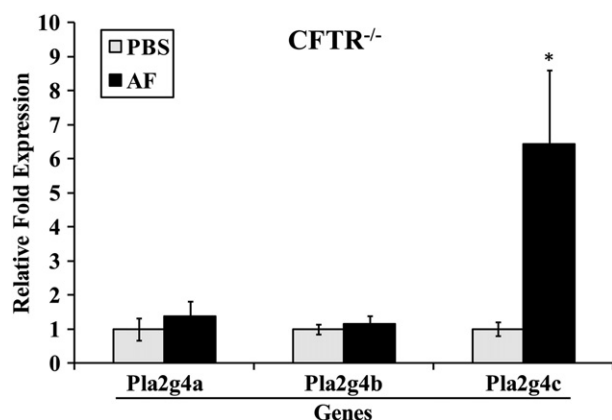


Fig. 2. Group IV cytosolic phospholipase A $_2$ s steady state mRNA levels in mouse lungs. CFTR $^{-/-}$  mice were sensitized/challenged with PBS or *Af* as described in Fig. 1. Real-time RT-PCR was then used to determine the steady state mRNA levels from whole lungs for the group IV PLA $_2$ s, Pla2g4a, Pla2g4b and Pla2g4c. \* indicates  $p \leq 0.05$  as compared to PBS-sensitized mice.

The alternate fate of AA is peroxidation by the lipoxygenase (LOX) family of enzymes leading to the production of lipoxins and leukotrienes (LTs). The LTs have physiological roles in innate immune responses in the lung and in the pathology of inflammatory diseases, such as asthma, allergic rhinitis and atherosclerosis [21]. Fig. 4A illustrates that the gene expression levels for Alox3, Alox5 and Alox5ap are not elevated in the CFTR $^{-/-}$  mice. We also evaluated other members of the LOX family: Alox12 (platelet 12(S)-LOX); Alox12b (Epidermis-type, 12(R)-LOX); Alox15 (12-LOX Leukocyte-type, human ortholog 15-LOX-A); Alox8 (8-LOX, human ortholog 15-LOX-B); and Alox12e (human ortholog pseudogene ALOX12P2) (Fig. 4A). These results demonstrate significant increases in the expression of most of the 12 and 15 LOXs in response to *Af* sensitization/challenge. The results in the CFTR $^{-/-}$  mice are consistent with our observations in C57BL/6 (Supp. Fig. 5A–B) and FVB mice as summarized in Table 2.

Based on these findings, we next evaluated the human orthologs, ALOX15 (Fig. 4B) and ALOX15B (Fig. 4C), in IB3.1 and S9 cells in response to various inflammatory mediators. We did not study Alox12e because this gene only exists in humans as a pseudogene (ALOX12P). Of note, several stimuli caused an upward trend in ALOX15 gene expression which occurred only in the IB3.1 cells (Fig. 4B). Similarly, we observed upward trends for various stimuli for ALOX15B gene expression primarily in the IB3.1 cells (Fig. 4C). The extremely low cellular abundance of both ALOX15 and ALOX15B mRNA in these cells leads to a high variance between levels of induction. Thus, despite the lack of statistical significance, we believe that these trends represent a true induction based on our experience with low abundance genes by using multiple PCR primer sets and corroboration by northern analysis.

## 4. Discussion

Eicosanoids constitute a diverse family of physiologically active fatty acids involved in regulating airway inflammation and reactivity, which are linked to diseases such as asthma and CF [1–3]. Recent studies have linked the ubiquitous fungus *A. fumigatus* (*Af*) with an increased prevalence in both asthmatics [22,23] and cystic fibrosis patients [24]. Therefore, we employed a mouse model that could mimic the pathology of allergen-induced asthma using an *Af* extract for sensitization and challenge. We hypothesized that potentially distinct enzymes in the eicosanoid pathway may display altered levels of gene expression in response to sensitization/challenge to this environmentally relevant allergen and the resulting inflammatory response, thus highlighting potentially novel therapeutic targets.

Our examination of the phospholipases (PLA $_2$ s) provides new insights into the role these enzymes may play in asthma and CF. To support our observations in the CFTR $^{-/-}$  mice, observed changes were validated in a human CF cell line alongside a cell line containing a functional CFTR as the only difference. Our data highlight potential new roles for Pla2g2c, Pla2g2d and Pla2g5 in allergen sensitization/challenge, based on the selective induction of these sPLA $_2$ s in the CFTR $^{-/-}$  mice (Fig. 1A). We have also corroborated the CF specific induction of PLA2G2D by

Table 1  
Fold change of PLA2G4C in S9 and IB3.1 cells.

|                 | LPS           | IFN $\gamma$  | TNF $\alpha$    | IL-1 $\beta$    | IL-2          | IL-3          | IL-4          | IL-6            | IL-10           | IL-13         | Af              |
|-----------------|---------------|---------------|-----------------|-----------------|---------------|---------------|---------------|-----------------|-----------------|---------------|-----------------|
| S9 <sup>a</sup> | 1.2 $\pm$ 0.1 | 0.9 $\pm$ 0.1 | 6.4 $\pm$ 1.0 * | 3.0 $\pm$ 0.2 * | 1.3 $\pm$ 0.1 | 1.4 $\pm$ 0.3 | 1.1 $\pm$ 0.1 | 1.4 $\pm$ 0.1 * | 1.2 $\pm$ 0.1 * | 1.0 $\pm$ 0.1 | 1.0 $\pm$ 0.1   |
| IB3.1           | 1.1 $\pm$ 0.1 | 0.8 $\pm$ 0.1 | 5.7 $\pm$ 1.7 * | 2.9 $\pm$ 0.1 * | 1.4 $\pm$ 0.3 | 1.4 $\pm$ 0.2 | 1.0 $\pm$ 0.1 | 1.3 $\pm$ 0.0 * | 1.1 $\pm$ 0.1   | 1.2 $\pm$ 0.1 | 1.4 $\pm$ 0.1 * |

\*  $p \leq 0.05$  by Student's t-test as compared to respective untreated cells.

<sup>a</sup> Originally published in Reference [20] — PLA2G4C data in S9 cells.

demonstrating that a number of inflammatory mediators can preferentially induce expression in the IB3.1 cells (LPS, IL-2, IL-6, IL-13, Af) (Fig. 1B). The unique expression in IB3.1 cells versus the corrected S9 may provide a relevant linkage to CF pathophysiology (Fig. 1B). Furthermore, the induction of PLA2G2D by IL-6 and IL-13 is a new observation in any cell type, since the only stimuli documented to induce this gene are IFN- $\gamma$  and LPS [25].

We next examined the group IV PLA<sub>2</sub>s, which have been directly linked to the liberation of AA as a consequence of the inflammatory response [26]. The vast majority of studies have focused on PLA2G4A/cPLA<sub>2</sub> $\alpha$ , which responds to a variety of stimuli and is regulated both at the transcriptional and post-translational levels [27]. Most surprisingly, we observed an induction of Pla2g4c in all mouse strains with no effect on Pla2g4a or Pla2g4b (Fig. 2 and Table 2). Furthermore, confirming that this allergic response is not antigen specific, Pla2g4c was also induced by OVA (Supp Fig. 3). This gene was similarly induced in both the IB3.1 and S9 cell lines by TNF- $\alpha$  and IL-1 $\beta$  (Table 1), implicating its potential importance to the inflammatory response. We believe that PLA2G4C responds to the allergic asthmatic response, with no appreciable contributions to this induction from the presence of the CFTR gene. We have also recently demonstrated that the induction of PLA2G4C specifically involves a promoter proximal element mediated by the transcription factors ATF-2/cJun, p65, and USF1/2 [20]. To date, the precise physiological role for PLA2G4C/cPLA<sub>2</sub> $\gamma$  has not been elucidated; it was first identified by orthology to PLA2G4A, to which it has ~30% overall sequence identity [28–32]. In addition to its PLA<sub>2</sub> activities, Yamashita et al. [29] have also reported that this enzyme displays coenzyme A (CoA)-independent transacylation and lysophospholipid (LPL) dismutase (LPLase/transacylase) activities and have suggested a possible role in fatty acid remodeling of phospholipids and the clearance of toxic lysophospholipids. Our new data in both allergic asthma and inflammation add impetus to further studies of this gene.

Another interesting result is the lack of a significant response from the enzymes on the cyclooxygenase branch of AA metabolism (Fig. 3 and Supp. Fig. 4), which, at least at the transcriptional level, implies that the prostanoids may have a limited role in this allergy model. These data directed our attention to the lipoxygenase branch where the literature [21,33] and the significant pharmaceutical investment in anti-LT therapies would argue that alterations in the expression of either Alox5 (5-LOX) or Alox5ap (FLAP) in response to Af could be expected. As with the prostanoid enzymes, we saw no changes with either of these enzymes or in the levels of Alox3 (Fig. 4A).

Af sensitization/challenge did, however, cause a significant increase in Alox15, Alox8 and the mouse specific Alox12e. Consistent with these data was the induction, only in IB3.1 cells, of the human ALOX15 (ortholog of mouse Alox15) by TNF- $\alpha$ , IL-3, and Af (Fig. 4B) and ALOX15B (ortholog of mouse Alox8) by IL-13 (Fig. 4C). The lack of response in S9 cells may be indicative of a more complex interplay between CFTR and ALOX15. ALOX15 has been previously shown to be regulated by IL-4 and IL-13 [34,35], however, no previous studies have linked induction of ALOX15 to TNF- $\alpha$  or IL-3. Our mouse data for Alox15 induction are also in line with recent studies by Andersson et al. [36] which demonstrated that Alox15<sup>-/-</sup> mice in a systemic OVA sensitization model had impaired airway inflammation, reduced levels of eosinophils, lymphocytes and macrophages in BAL fluid along with lower levels of the T<sub>H</sub>2 cytokines (IL-4, IL-5 and IL-13) and tissue eosinophils. Our data and that of Andersson et al. [36] would imply that Alox15 inhibition may provide an alternative therapeutic target for asthma and CF patients. This would also be consistent with studies that have demonstrated an increased level of 15-LOX metabolites in asthmatics, where 15(S)-HETE levels in BALF were elevated and associated with tissue eosinophil numbers, sub-membrane thickness and the observation that severe asthmatics presenting with persistent airway eosinophils exhibit high levels of 15(S)-HETE in BALF [37,38].

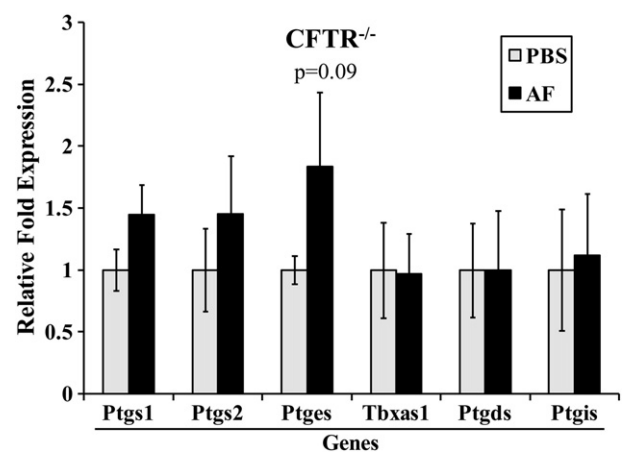


Fig. 3. Gene expression analysis of enzymes involved in prostanoid synthesis in mouse lungs. CFTR<sup>-/-</sup> mice were sensitized/challenged as described in Fig. 1, and real-time RT-PCR on whole lung mRNA was performed for Ptgs1, Ptgs2, Ptges, Tbxas1, Ptgds, and Ptgis. Data points are the means of  $2^{-\Delta\Delta CT} \pm$  SEM ( $7 \leq n \leq 10$ ).

Table 2

Fold change of phospholipase A2 and lipoxygenase gene expression in *Af* sensitization/challenge, compared to PBS sensitized mice.

|                             | CFTR <sup>-/-</sup> | C56BL/6              | FVB       |
|-----------------------------|---------------------|----------------------|-----------|
| <i>Pla2g2c</i>              | 2.3±0.4 *           | 0.9±0.1 <sup>a</sup> | –         |
| <i>Pla2g2d</i>              | 3.2±0.9 *           | 1.4±0.3 <sup>a</sup> | 0.7±0.2   |
| <i>Pla2g2e</i>              | 1.2±0.2             | 1.7±0.2 *            | 1.5±0.3   |
| <i>Pla2g5</i>               | 3.6±0.5 *           | 1.9±0.3 <sup>a</sup> | 0.9±0.2   |
| <i>Pla2g10</i>              | 1.1±0.3             | 1.0±0.2 <sup>a</sup> | –         |
| <i>Pla2g12b</i>             | 1.1±0.2             | 0.5±0.1 *            | –         |
| <i>Pla2g4a</i>              | 1.4±0.4             | 1.2±0.1              | 1.0±0.2   |
| <i>Pla2g4b</i>              | 1.2±0.2             | 0.9±0.1              | –         |
| <i>Pla2g4c</i>              | 6.4±2.2 *           | 25.0±2.8 *           | 2.6±0.7   |
| <i>Ptgs1</i>                | 1.4±0.2             | 0.9±0.3              | –         |
| <i>Ptgs2</i>                | 1.5±0.5             | 0.7±0.3              | –         |
| <i>Ptgs</i>                 | 1.8±0.6             | 2.2±0.7              | –         |
| <i>Tbxas1</i>               | 1.0±0.3             | 1.1±0.3              | –         |
| <i>Ptgds</i>                | 1.0±0.5             | 0.3±0.1              | –         |
| <i>Ptgis</i>                | 1.1±0.5             | 0.7±0.3              | –         |
| <i>Aloxe3</i>               | 1.2±0.3             | 0.7±0.1 *            | –         |
| <i>Alox5</i>                | 0.9±0.1             | 1.2±0.2              | –         |
| <i>Alox5ap</i>              | 1.2±0.3             | 1.9±0.4              | –         |
| <i>Alox12</i>               | 0.6±0.1             | 0.5±0.1 *            | 0.4±0.1 * |
| <i>Alox12b</i>              | 1.7±0.3 *           | 0.7±0.1              | –         |
| <i>Alox15</i>               | 4.2±0.6 *           | 30.1±6.1 *           | 3.1±0.8 * |
| <i>Alox8</i>                | 3.6±0.8 *           | 4.7±0.6 *            | 0.8±0.3   |
| <i>Alox12e</i> <sup>b</sup> | 9.2±1.8 *           | 20.0±2.7 *           | 3.7±1.1 * |

Values represent means of  $2^{-\Delta\Delta CT} \pm SEM$ .

\*  $p \leq 0.05$  by Student's t-test.

<sup>a</sup> Originally published in Reference [20].

<sup>b</sup> Mouse ortholog of the human pseudogene ALOX12P.

Other than evidence that the ALOX15B gene may be regulated by its own product [39], very little is known about this enzyme's regulation, thus making the induction in the *Af*-treated mice and by IL-13 in the IB3.1 cells an intriguing observation. In addition to its role in the synthesis of pro-inflammatory products, 15-lipoxygenases also participate through transcellular biosynthesis in the production of anti-inflammatory bioactive lipid mediators of resolution, the lipoxins [40–43]. Lipoxins, LXA<sub>4</sub> and LXB<sub>4</sub> being the main components, are lipid mediators generated from AA that act to reduce inflammation and promote resolution. Lipoxins are generated through the combined action of 5- and 15-lipoxygenases during cell–cell interactions. Recent data have also demonstrated that lipoxin concentrations in CF patient's airway fluid are significantly suppressed when compared to other inflammatory lung conditions [40].

We therefore hypothesize that the coupled induction of both Pla2g4c and the lipoxygenases in our mouse models of *Af* sensitization/challenge provides for the generation of both arachidonate and downstream lipoxygenase metabolites as potent mediators in the pathology of ABPA, asthma and CF. To this end, we have identified a number of genes in the eicosanoid pathway that display altered gene expression that may be associated with ABPA, allergic asthma or CF. The results have also helped to highlight a PLA2G4C→lipoxygenase axis where the downstream metabolites can act as potentially important mediators in the inflammatory response in these diseases. Furthermore, the altered expression of these genes provides potential identifiers and therapeutic targets that are specifically linked to *Aspergillus* sensitization and/or cystic fibrosis.

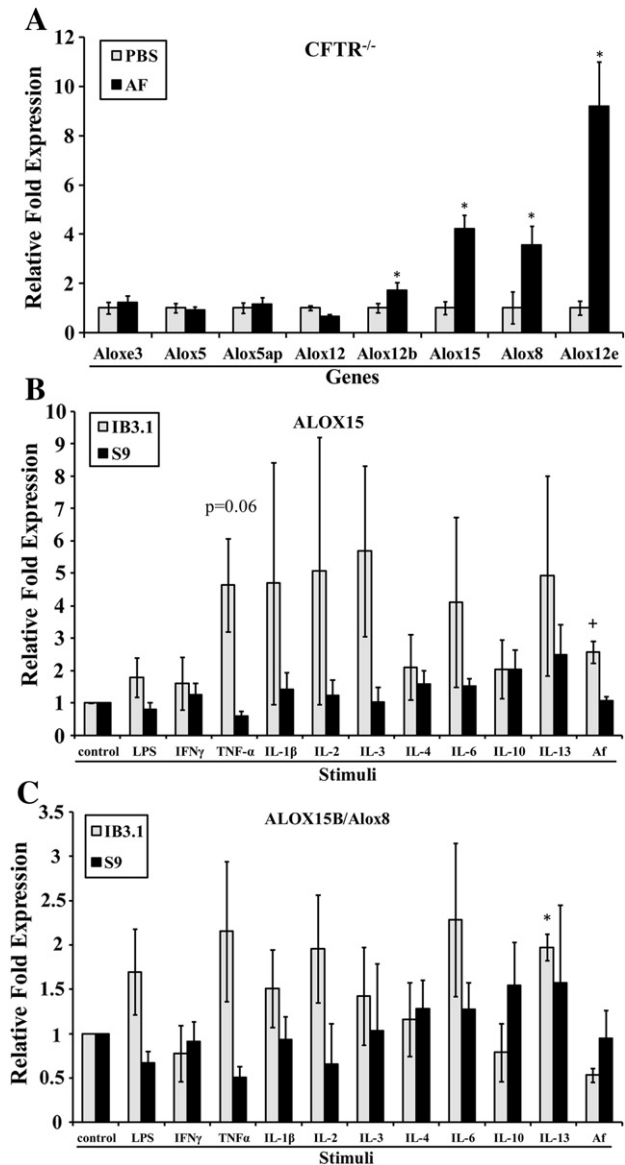


Fig. 4. Gene expression analysis of enzymes in the lipoxygenase pathway in mouse lungs and human cell lines. (A) CFTR<sup>-/-</sup> mice were sensitized/challenged with PBS or *Af*, and whole lung mRNA was analyzed by real-time RT-PCR for lipoxygenase gene expression (Aloxe3, Alox5, Alox5ap, Alox12, Alox12b, Alox15, Alox8, and Alox12e). Data points are the means of  $2^{-\Delta\Delta CT} \pm SEM$  ( $7 \leq n \leq 10$ ). \* indicates  $p \leq 0.05$  as compared to PBS-sensitized mice. (B) Steady state mRNA levels of human ALOX15 in response to the indicated stimuli in S9 or IB3.1 cells. Data points are the means of  $2^{-\Delta\Delta CT} \pm SEM$  ( $n=3$ ). (C) Steady state mRNA levels of human ALOX15B in response to the indicated stimuli in S9 or IB3.1 cells. Data points are the means of  $2^{-\Delta\Delta CT} \pm SEM$  ( $n=3$ ). \* and + indicate  $p \leq 0.05$  as compared to control and S9 cells, respectively.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jcf.2012.08.014>.

## Conflict of interest

The authors have no conflicts of interest to disclose.

## Role of funding source

The funding source had no role in the study design, in the writing of the report or in the decision to submit the paper for publication.

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**Supplementary Table 1.** List of primers for real-time PCR.

| Gene                          | Sequence                                                       |
|-------------------------------|----------------------------------------------------------------|
| <i>Ppia</i>                   | GCG GCA GGT CCA TCT ACG<br>GCC ATC CAG CCA TTC AGT CT          |
| <i>IL-1<math>\beta</math></i> | GCC TGT GTT TTC CTC CTT GC<br>CAG TGC GGG CTA TGA CCA A        |
| <i>IL-2</i>                   | CAC CCT TGC TAA TCA CTC CTC A<br>CTG CTG TGC TTC CGC TGT AG    |
| <i>IL-3</i>                   | TCC TGA TGC TCT TCC ACC TG<br>CCA CTT CTC CTT GGC TTT CC       |
| <i>IL-4</i>                   | GCG ACA AAA ATC ACT TGA GAG AG<br>GCA CCT TGG AAG CCC TAC AG   |
| <i>IL-6</i>                   | TGG GAC TGA TGC TGG TGA CA<br>CTA TTT CCA CGA TTT CCC AGA G    |
| <i>IL-10</i>                  | CCA GTA CAG CCG GGA AGA CA<br>TTT CTG GGC CAT GCT TCT CT       |
| <i>IL-13</i>                  | TTG CTT GCC TTG GTG GTC TC<br>CCT CTG GGT CCT GTA GAT GGC      |
| <i>IFN<math>\gamma</math></i> | CCA AGC GGC TGA CTG AAC TC<br>GCC AGT TAC TAC CTG ACA CAT TCG  |
| <i>TGF-<math>\beta</math></i> | AGA CAG CAA AGA TAA CAA ACT CCA C<br>GCC GCA CAC AGC AGT TCT T |
| <i>Eotaxin</i>                | GCT CAC GGT CAC TTC CTT CAC<br>GTG CTT TGT GGC ATC CTG G       |
| <i>KC</i>                     | GCA CCC AAA CCG AAG TCA TAG                                    |

|                |                                |
|----------------|--------------------------------|
|                | CAG ACA GGT GCC ATC AGA GC     |
| <i>GM-CSF</i>  | CAA GTT ACC ACC TAT GCG GAT TT |
|                | CAT TAC GCA GGC ACA AAA GC     |
| <i>MIP-2</i>   | TCC CTC AAC GGA AGA ACC AA     |
|                | TAA CAA CAT CTG GGC AAT GGA A  |
| <i>Ptgs1</i>   | GCT CAC AGG AGA GAA GGA GAT G  |
|                | GGA GCC CCC ATC TCT ATC ATA C  |
| <i>Ptgs2</i>   | GGC TTC GGG AGC ACA ACA        |
|                | CAA TGC GGT TCT GAT ACT GGA    |
| <i>Ptges</i>   | TTA GAG GTG GGC AGG TCA GAG    |
|                | CCA CTC GGG CTA AGT GAG AC     |
| <i>Tbxas1</i>  | AAG AGG AAG TAT CCC CAG AAC C  |
|                | TGT CCA GAT ACG GCA GAC CTT    |
| <i>Ptgds</i>   | GTC AGT CAG AGG GCT GGT CAC    |
|                | GGA CTC TTA TCC TTC TCC TCA CG |
| <i>Ptgis</i>   | TTC CAT CCC TAT GCC ATC TTC    |
|                | TGA GCA GGG CGT GTA GGA        |
| <i>Aloxe3</i>  | CGG TTC CCA GAG TTG TCA TCC    |
|                | AAG CCC GCC AAG AAT GTT ATC    |
| <i>Alox5</i>   | TGT TCC CAT TGC CAT CCA G      |
|                | CAC CTC AGA CAC CAG ATG CG     |
| <i>Alox5ap</i> | ATC AAG AGG CTG TGG GCA AC     |
|                | TAG ACC CGC TCA AAG GCA AG     |
| <i>Alox12</i>  | TGA CGA TGG AGA CCG TGA TG     |
|                | GCT TTG GTC CTT GGG TCT GA     |

|                 |                                                                |
|-----------------|----------------------------------------------------------------|
| <i>Alox12b</i>  | GCA CTT TGG TCC TGA TGG C<br>CCT CGT GGC TGT AGA ACT CC        |
| <i>Alox12e</i>  | CCT TTT TCC CCT GCT ACA GTT G<br>CCC CAC CGA TAC ACA TTC CT    |
| <i>Alox15</i>   | AAA GGC ACT CTG TTT GAA GCG<br>CAC CAA GTG TCC CCT CAG AAG     |
| <i>Alox8</i>    | CCT GCC CAG CGA TGA CAC<br>CCG AAT GTG AGG AAT CAA TAG C       |
| <i>Pla2g2c</i>  | CCA ACC CAT CTT GAA TGC CTA CC<br>GGA GTT TGT CCC TGC CAC A    |
| <i>Pla2g2d</i>  | AAC CTG AAC AAG ATG GTC ACA CAC<br>GGT GGG CAT AGC AAC AAT CAT |
| <i>Pla2g2e</i>  | CAG TGG ACG AGA CGG ATT GG<br>CAG GTT GTG GCG AAA GCA G        |
| <i>Pla2g5</i>   | CTA CTG CCT GCC GAG AAA CC<br>ACA CAT CAG GAA TAC AGC AGA GG   |
| <i>Pla2g10</i>  | GAA ATA CCT CTT CTT CCC CTC C<br>CAG GTG GCT TTA GCA CTT GG    |
| <i>Pla2g12b</i> | GGT GCC TCC ACT CAA TCT GC<br>GCT GCC CGC TGA CTG TTC          |
| <i>Pla2g4a</i>  | CAG CCA CAA CCC TCT CTT ACT TC<br>CGG CAT TGA CCT TTT CCT TC   |
| <i>Pla2g4b</i>  | GCA CAA GGA CCA CTA TGA GAA TC<br>ACC ACC CTA AAA GTG CCC TC   |
| <i>Pla2g4c</i>  | CAC AAA CGC AGT CCC AAG G                                      |

|                |                                 |
|----------------|---------------------------------|
|                | AGA CCC CTG CGA GGT ATG TG      |
| <i>PPIA</i>    | CAT CCT AAA GCA TAC GGG TCC     |
|                | GCT GGT CTT GCC ATT CCT G       |
| <i>PLA2G2D</i> | GCC CTC CAC TGT CCT AAG ATA AAG |
|                | TTC ACC CTG AGC CAA TGC C       |
| <i>PLA2G5</i>  | GCG GCT GGA GGA GAA GG          |
|                | GAG CAG AGG ATG TTG GGA AAG     |
| <i>PLA2G4C</i> | CAC CTG GCT GAC TGA GAT GCT     |
|                | CGC AAA TGC CTG TTT TCT TC      |
| <i>PTGS1</i>   | GAG TTT GTC AAT GCC ACC TTC A   |
|                | TGG GGC AAT CTT TAG GCA C       |
| <i>PTGES</i>   | GCC GCC GTG GCT ATA CC          |
|                | GGT TCC CAT CAG CCA CTT C       |
| <i>TBXAS1</i>  | AGA GCA CGG TTC CCA TAA GG      |
|                | CCA AGA GAG CCA CTG ACA GG      |
| <i>PTGIS</i>   | CAG ACG ACC ACT CTC CCA CAG     |
|                | GGA AGA GGA GGA GGC GGT         |
| <i>ALOX15</i>  | GCA AGG AGA CAG AAC TCA AGG TG  |
|                | TCA GGC AGG CTC AGG ACG         |
| <i>ALOX15B</i> | GCC CAA ACA GCC CCA TC          |
|                | CAG GCA GAC ACA GGA GAG AAT AG  |

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All sequences are listed in the 5'→3' direction for each primer with the forward strand primer listed first followed by the reverse strand primer.

**Supplemental Table 2.** Orthologous mouse and human genes in the eicosanoid pathway.

| <b>Mouse Gene Name</b>                       | <b>Mouse Gene Symbol</b> | <b>Mouse NCBI mRNA accession #</b> | <b>Human Gene Name</b>                       | <b>Human Gene Symbol</b> | <b>Human NCBI mRNA accession #</b> |
|----------------------------------------------|--------------------------|------------------------------------|----------------------------------------------|--------------------------|------------------------------------|
| cPLA <sub>2</sub> α                          | Pla2g4a                  | NM_008869                          | cPLA <sub>2</sub> α                          | PLA2G4A                  | NM_024420                          |
| cPLA <sub>2</sub> β                          | Pla2g4b                  | NM_145378                          | cPLA <sub>2</sub> β                          | PLA2G4B                  | NM_001114633                       |
| cPLA <sub>2</sub> γ                          | Pla2g4c                  | NM_001168504                       | cPLA <sub>2</sub> γ                          | PLA2G4C                  | NM_003706                          |
| sPLA <sub>2</sub> -IIC                       | Pla2g2c                  | NM_008868                          | sPLA <sub>2</sub> -IIC                       | PLA2G2C                  | NM_001105572                       |
| sPLA <sub>2</sub> -IID                       | Pla2g2d                  | NM_011109                          | sPLA <sub>2</sub> -IID                       | PLA2G2D                  | NM_012400                          |
| sPLA <sub>2</sub> -IIE                       | Pla2g2e                  | NM_012044                          | sPLA <sub>2</sub> -IIE                       | PLA2G2E                  | NM_001080490                       |
| sPLA <sub>2</sub> -V                         | Pla2g5                   | NM_012044                          | sPLA <sub>2</sub> -V                         | PLA2G5                   | NM_000929                          |
| sPLA <sub>2</sub> -X                         | Pla2g10                  | NM_011987                          | sPLA <sub>2</sub> -X                         | PLA2G10                  | NM_003561                          |
| sPLA <sub>2</sub> -XIIB                      | Pla2g12b                 | NM_023530                          | sPLA <sub>2</sub> -XIIB                      | PLA2G12B                 | NM_032562                          |
| COX-1, Prostaglandin endoperoxide synthase 1 | Ptgs1                    | NM_008969                          | COX-1, Prostaglandin endoperoxide synthase 1 | PTGS1                    | NM_000962                          |
| COX-2, Prostaglandin endoperoxide synthase 2 | Ptgs2                    | NM_011198                          | COX-2, Prostaglandin endoperoxide synthase 2 | PTGS2                    | NM_000963                          |
| PGI2 (prostacyclin) synthase                 | Ptgis                    | NM_008968                          | PGI2 (prostacyclin) synthase                 | PTGIS                    | NM_000961                          |
| mPGES-1, PGE synthase                        | Ptges                    | NM_022415                          | mPGES-1, PGE synthase                        | PTGES                    | NM_004878                          |
| L-PGDS, PGD2 synthase                        | Ptgds                    | NM_008963                          | L-PGDS, PGD2 synthase                        | PTGDS                    | NM_000954                          |
| TXAS, TXA synthase 1 (platelet)              | Tbxas1                   | NM_011539                          | TXAS, TXA synthase 1 (platelet)              | TBXAS1                   | NM_001061                          |
| eLOX3                                        | Aloxe3                   | NM_011786                          | eLOX3                                        | ALOXE3                   | NM_001165960                       |
| Flap, 5-lipoxygenase activating protein      | Alox5ap                  | NM_009663                          | FLAP, 5-lipoxygenase activating protein      | ALOX5AP                  | NM_001629                          |
| 8-LOX                                        | Alox8                    | NM_009661                          | 15-LOX-B, 15-LOX-2                           | ALOX15B                  | NM_001141                          |
| 12S-LOX, Platelet 12-LOX, 12S-type           | Alox12                   | NM_007440                          | 12S-LOX, Platelet 12-LOX, 12S-type           | ALOX12                   | NM_000697                          |
| 12R-LOX, Epidermis-type 12R-type             | Alox12b                  | NM_009659                          | 12R-LOX, Epidermis-type 12R-type             | ALOX12B                  | NM_001139                          |
| 5-LOX                                        | Alox5                    | NM_009662                          | 5-LOX                                        | ALOX5                    | NM_000698                          |
| 12-LOX Leukocyte-type                        | Alox15                   | NM_009660                          | 15-LOX, 15-LOX-1                             | ALOX15                   | NM_001140                          |
| 12-LOX Epidermal-type                        | Alox12e                  | NM_145684                          | 12-LOX pseudogene 2                          | ALOX12P2                 | NR_002710                          |

List of mouse genes used in this study with orthologous human genes and corresponding accession numbers for relevant mRNA sequences.