Reduced 15-lipoxygenase 2 and lipoxin A_4/leukotriene B_4 ratio in children with cystic fibrosis

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ABSTRACT Airway disease in cystic fibrosis (CF) is characterised by impaired mucociliary clearance, persistent bacterial infection and neutrophilic inflammation. Lipoxin A_4 (LXA_4) initiates the active resolution of inflammation and promotes airway surface hydration in CF models. 15-Lipoxygenase (LO) plays a central role in the ''class switch'' of eicosanoid mediator biosynthesis from leukotrienes to lipoxins, initiating the active resolution of inflammation. We hypothesised that defective eicosanoid mediator class switching contributes to the failure to resolve inflammation in CF lung disease.

Using bronchoalveolar lavage (BAL) samples from 46 children with CF and 19 paediatric controls we demonstrate that the ratio of LXA_4 to leukotriene B_4 (LTB_4) is depressed in CF BAL ($p<0.01$), even in the absence of infection ($p<0.001$).

Furthermore, 15-LO2 transcripts were significantly less abundant in CF BAL samples ($p<0.05$). In control BAL, there were positive relationships between 15-LO2 transcript abundance and LXA_4/LTB_4 ratio ($p=0.01$, $r=0.66$) and with percentage macrophage composition of the BAL fluid ($p<0.001$, $r=0.82$), which were absent in CF.

Impoverished 15-LO2 expression and depression of the LXA_4/LTB_4 ratio are observed in paediatric CF BAL. These observations provide mechanistic insights into the failure to resolve inflammation in the CF lung.

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Introduction

Cystic fibrosis (CF) is caused by mutation of the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel and characterised by impaired mucociliary clearance, persistent bacterial infection and neutrophil mediated inflammation.

Sequential biosynthesis of eicosanoid classes in inflammatory exudates (termed "class switching") directs the temporal progression of acute inflammation from initiation to propagation and finally to active resolution [1]. Prostaglandins initiate the inflammatory response. Leukotrienes follow and are typified by leukotriene B_4 (LTB_4) which amplifies and propagates inflammation [1]. LTB_4 and interleukin (IL)-8 are both potent neutrophil chemoattractants [2, 3] and negatively correlate with pulmonary function in CF [4, 5]. Lipoxin A_4 (LXA_4) biosynthesis initiates the active resolution phase of inflammation [6], followed by biosynthesis of the resolvins and protectins.

LXA_4 inhibits neutrophil effector functions [7] and counter-regulates the effector functions of LTB_4, most notably inhibiting LTB_4-induced neutrophil transmigration [8–10]. LXA_4 also suppresses production of IL-8 by leukocytes and bronchial epithelial cells [11–13]. Mice treated with analogues of LXA_4 and subsequently challenged with *Pseudomonas aeruginosa* contained the bacterial challenge more effectively [14]. LXA_4 augments airway epithelial innate defence by stimulating tight junction formation [15], enhancing calcium-activated chloride secretion [16], and by restoration of airway surface liquid height in CF airway epithelial cells [17]. LXA_4 concentration in CF bronchoalveolar lavage (BAL) has been variously reported as significantly suppressed or not significantly different from controls [14, 18].

LXA_4 is biosynthesised in the respiratory tract by transcellular cooperation of neutrophils [19], eosinophils [20], alveolar macrophages [21] or airway epithelial cells [22], each expressing different lipoxygenase (LO) enzymes (fig. 1) [2, 23]. Upregulation of 15-LO activity favours LXA_4 biosynthesis at the expense of leukotriene biosynthesis [1, 24]. This occurs both as a result of 15-LO product, 15-hydroxyeicosatetraenoic acid (HETE), competing for catalytic sites at the 5-LO enzyme and thereby reducing the formation of 5(S) HETE, and by competition for the common biosynthetic intermediate leukotriene A_4 [1, 22–24].

We hypothesised that "eicosanoid mediator class switching" is defective in CF and may play a mechanistic role in the failure to resolve inflammation in CF lung disease. We measured eicosanoids associated with the "class switch" [1] from propagation (leukotrienes) to resolution of inflammation (lipoxins), and the relative expression of mRNA transcripts for eicosanoid synthetic enzymes (5-LO, 12-LO and 15-LO1/2 and leukotriene A_4 hydrolase (LTA_4H)) in BAL from children with CF and compared this with paediatric

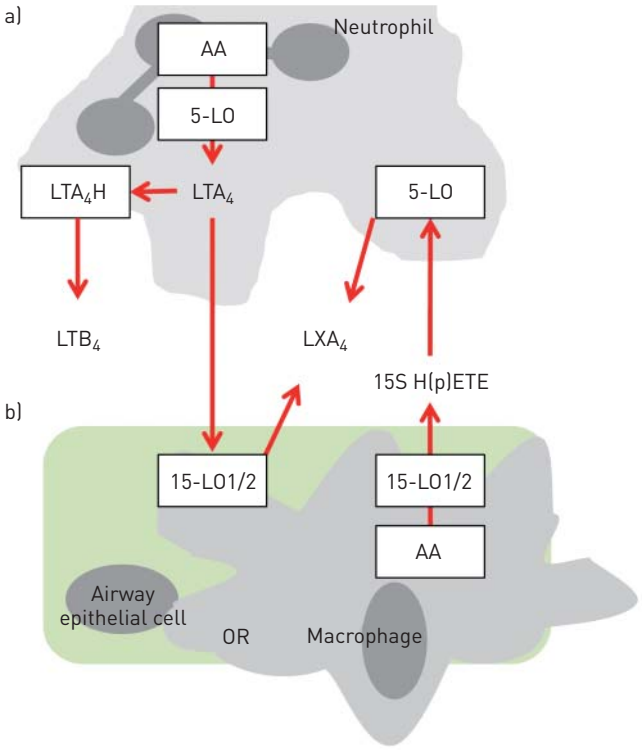

FIGURE 1 An illustration of lipoxin A_4 (LXA_4) biosynthesis pathways involving transcellular cooperation in the airways. a) The neutrophil donates the leukotriene A_4 (LTA_4) intermediate formed by the action of 5-lipoxygenase (LO) on arachidonic acid (AA) to the acceptor airway epithelial cell or alveolar macrophage, whereby 15-LO1/2 (15-LO isoform 1 or 2) catalyses LXA_4 formation. Leukotriene B_4 (LTB_4) is also generated from the LTA_4 intermediate by the action of leukotriene A_4 hydrolase (LTA_4H). b) Airway epithelial cell or alveolar macrophage 15-LO1/2 activity catalyses the conversion of AA to 15(S)-hydroperoxyeicosatetraenoic acid 15(S) H(p)ETE, which is donated to the acceptor neutrophil and converted to LXA_4 by 5-LO catalysis.

controls. In addition, we conducted subgroup analysis based on the presence or absence of microbial pathogens ($>10^4$ CFU·mL^{-1}) detected in the BAL fluid.

Methods

Study population

Children with CF were recruited through SHIELD CF (Study of Host Immunity and Early Lung Disease in Children with CF), a longitudinal study established around the preschool surveillance bronchoscopy programmes at Our Lady's Children Hospital, Crumlin (OLCHC), the National Children's Hospital, Tallaght and University Hospital Limerick, Limerick, Ireland. Children without CF undergoing bronchoscopy for clinical reasons were recruited as paediatric controls (see the online supplementary material for indications and treatment characteristics). Assessments were undertaken when the children were in a stable clinical condition. Children were excluded from the analysis if they were taking leukotriene receptor antagonists or treatment doses of antibiotics in the 2 weeks prior to bronchoscopy. Ethical approval for this study was granted by the research ethics committee at each hospital and written informed consent was obtained from the parents of all participating subjects. Bronchoscopy, BAL collection and processing to obtain a supernatant and cell pellet were performed as previously described (online supplementary material) [25].

Measurements

An aliquot of BAL was sent to the microbiology lab at OLCHC where testing, including conventional bacterial and viral studies, was performed according to Clinical Pathology Accreditation guidelines (online supplementary material). Samples were considered to have positive microbial culture if one or more pathogen was detected on viral studies or cultured at a concentration of $>10^4$ CFU·mL^{-1}.

LXA_4 and LTB_4 concentrations were measured by ELISA (Oxford Biomedical Research, Rochester Hills, MI, USA and Cayman Chemical, Ann Arbor, MI, USA) (for specificities of antisera see online supplementary table E1). IL-8 was measured by ELISA using mouse anti-IL8 capture antibody and biotinylated goat anti-IL8 detection antibody (R&D Systems, Abingdon, UK). Neutrophil elastase (NE) activity was measured as described previously (online supplementary material) [25]. Total and differential cell counts were obtained by the trypan blue exclusion method and light microscopy (for further detail see the online supplementary material).

Total RNA was extracted from the BAL cell pellets using RNAqueous kit (Ambion Inc., Austin, TX, USA). RNA was reverse transcribed using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Dun Laoghaire, Ireland). Real-time PCR was performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems) using TaqMan probes to detect genes coding for arachidonate 5-lipoxygenase (ALOX5), arachidonate 12-lipoxygenase (ALOX12), arachidonate 15-lipoxygenase (ALOX15), arachidonate 15-lipoxygenase type B (ALOX15B) and LTA_4H (Applied Biosystems) and 18S ribosomal RNA used as internal control. The threshold cycle for each target gene was normalised using the Ct of the endogenous control (18S). Relative fold expression for each target was calculated as ($2^{(-\Delta\Delta Ct)}$) in comparison to a reference patient.

Statistical analysis

Graphpad prism (GraphPad Software, Inc., La Jolla, CA, USA) was used to perform statistical analyses. Results are presented as mean$\pm$SD or mean$\pm$SEM. The groups were compared using Fisher's exact test for categorical variables and unpaired t-test for continuous variables. Correlations were analysed using the Spearman test. No correction was made for multiple comparisons. Results were reported as statistically significant with p-values <0.05, <0.01 and <0.001.

Results

51 BAL samples from children with CF and 22 from paediatric control patients were assessed for eligibility in this study. Five children with CF and three control children were excluded because of leukotriene receptor antagonist treatment or recent use of treatment dose antibiotics. CF and control children did not differ significantly in age, sex or BAL neutrophil count (table 1). Control children with positive BAL microbial culture were younger than control children with negative BAL microbial culture. Children with CF with positive BAL microbial culture were older than children with CF with negative BAL microbial culture. Children with CF with positive BAL samples had higher mean neutrophil counts and more frequently detectable NE than culture negative CF samples (table 1). The most common bacterial pathogens cultured were *Haemophilus influenzae*, *Staphylococcus aureus* and *Streptococcus pneumonia*. *P. aeruginosa* $>10^4$ CFU·mL^{-1} was identified in two CF and one control BAL sample. Viral studies were negative for all BAL samples (CF and controls).

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TABLE 1 Population characteristics

	Non-CF control			CF		
	Culture negative	Culture positive	All controls	Culture negative	Culture positive	All CF
Procedures	7	12	19	24	22	46
Subjects			19			34
Mean±SD age years	8.2±4.3**	2.7±1.6**	4.5±3.6	2.7±1.5*	3.8±1.4*	3.2±1.6
Male sex	2 (28.6)	8 (66.7)	10 (52.6)	12 (50)	12 (54.5)	24 (52.2)
Neutrophils $\times 10^5 \cdot mL^{-1}$	0.8±0.6#	8.1±4.0	5.7±2.8	3.5±1.0**	12.8±2.8**, #	8.0±1.6
Neutrophil elastase	3 (42.9)	5 (41.7)	8 (42.1)	9 (37.5)¶	17 (77.3)¶	26 (56.5)

Data are presented as n, n (%) or mean±SEM, unless otherwise stated. Data are presented for the complete control and cystic fibrosis (CF) groups, and separately for subgroups based on the presence (culture positive) or absence (culture negative) of $>10^4$ CFU·mL^{-1} pathogenic bacteria cultured from the BAL specimen. *: p<0.05; **: p<0.01 within group unpaired comparison of subgroups. #: p<0.05 between group unpaired t-test comparison of subgroups; ¶: p<0.01 within group comparison, Fisher's exact test.

We found no significant differences between control and CF samples in mean LXA_4 or LTB_4 concentration (fig. 2a and c). LXA_4 and LTB_4 concentrations adjusted for neutrophil and macrophage count and examined by microbial culture result are presented in online supplementary figure E1. IL-8 was higher in CF samples than in control samples (1198±149 ng·mL^{-1} *versus* 379±110 ng·mL^{-1}, p<0.01) (fig. 2e).

Comparison of culture negative and positive control and CF samples revealed no significant difference in LXA_4 concentration (fig. 2b). LTB_4 concentration was significantly higher in infected samples within a group (control with positive microbial culture 155±40 pg·mL^{-1} *versus* control with negative culture 26±12 pg·mL^{-1}, p<0.05; and CF with positive microbial culture 1383±454 pg·mL^{-1} *versus* CF with negative culture 156±35 pg·mL^{-1}, p<0.01) (fig. 2d). IL-8 was higher in CF BAL with negative culture than in control BAL with negative culture (817±178 ng·mL^{-1} *versus* 110±28 ng·mL^{-1}, p<0.05) (fig. 2f). CF BAL samples with positive microbial culture had higher IL-8 concentrations than either CF samples with negative culture (1614±215 ng·mL^{-1} *versus* 817±178 ng·mL^{-1}, p<0.01) or control samples with negative culture (1614±215 ng·mL^{-1} *versus* 536±159 ng·mL^{-1}, p<0.01).

To evaluate the relative concentrations of these mediators in BAL we calculated LXA_4/LTB_4 and LXA_4/IL-8 ratios. Children with CF had lower LXA_4/LTB_4 ratios in BAL fluid compared with control children (2.9±0.7 *versus* 7.1±1.9, p<0.01) (fig. 3a). Among control samples, the LXA_4/LTB_4 ratio in sterile samples was significantly higher (13.1±3.6 *versus* 3.1±3.6, p<0.01) than that measured in BAL samples from which pathogens were cultured (fig. 3b). In CF BAL, the LXA_4/LTB_4 ratio was uniformly depressed and did not vary with infection status (p=0.98). The LXA_4/LTB_4 ratio measured in CF samples with negative microbial culture was significantly depressed in comparison to control BAL with negative culture (2.9±0.7 *versus* 13.1±3.6, p<0.001).

The LXA_4/IL-8 ratio was significantly higher in control BAL than in CF BAL ($3.5\times10^{-3}\pm1.7\times10^{-3}$ *versus* $0.5\times10^{-3}\pm0.1\times10^{-3}$, p<0.01) (fig. 3c). The LXA_4/IL-8 ratio was significantly higher in control samples with negative microbial culture than in controls with positive BAL microbial culture ($7.9\times10^{-3}\pm4.2\times10^{-3}$ *versus* $0.9\times10^{-3}\pm0.2\times10^{-3}$, p<0.05) (fig. 3d). The LXA_4/IL-8 ratio measured in CF BAL with negative microbial culture was significantly lower compared with control BAL with negative microbial culture ($0.6\times10^{-3}\pm0.2\times10^{-3}$ *versus* $7.9\times10^{-3}\pm4.2\times10^{-3}$, p<0.01). In CF BAL, the LXA_4/IL8 ratio was uniformly depressed and the correlation with infection seen in control BAL was absent.

14 control (seven with negative and seven with positive microbial culture) and 11 CF (six with negative and five with positive microbial culture) BAL cell pellets with a similar distribution of infection, and neutrophil (p=0.95) and macrophage (p=0.71) compositions were selected from the original sample set to study the expression of genes involved in LXA_4 and LTB_4 biosynthesis in the airway lumen (fig. 4e and f). The cellular fraction obtained from the airway lumen contained the full repertoire of transcripts required for LXA_4 biosynthesis (fig. 4). ALOX15B mRNA was significantly less abundant in CF samples compared to control samples (0.44±0.13 *versus* 2.63±0.92 fold expression, p<0.05) (fig. 4a). A nonsignificant trend towards lower ALOX15 expression was observed in CF BAL than in control BAL (1.92±0.49 *versus* 5.51±1.89 fold expression, p=0.09) (fig. 4b). ALOX5 and LTA4H mRNA abundance did not significantly differ between control and CF samples (fig. 4c and d). ALOX12 mRNA was measurable in only six out of 14 control samples and in four out of 11 CF samples.

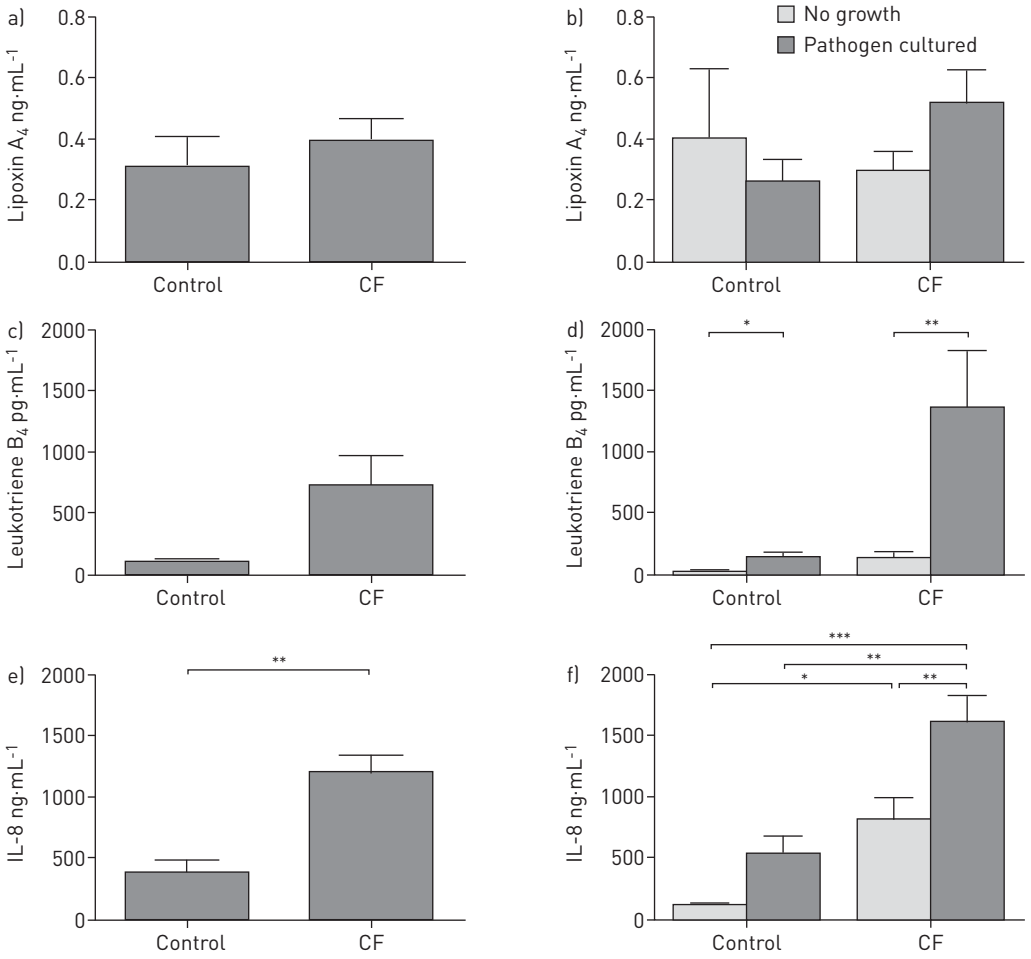

FIGURE 2 Baseline inflammatory parameters and their relationship to pulmonary infection. Comparison of mean a) lipoxin A_4, c) leukotriene B_4 and e) interleukin (IL)-8 values measured in control and cystic fibrosis (CF) bronchoalveolar lavage (BAL) samples. Comparison of mean b) lipoxin A_4, d) leukotriene B_4 and f) IL-8 in subgroups of the control and CF cohorts based on the presence or absence of $>10^4$ CFU·mL^{-1} pathogenic bacteria cultured from the BAL specimen. Error bars represent SEM. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$, t-test.

In control BAL samples there was a significant relationship between ALOX15B mRNA abundance in the cell pellet and LXA_4/LTB_4 ratio ($r=0.66$, $p=0.01$) (fig. 5a). In CF samples this correlation was absent ($p=0.31$) (fig. 5b). Neutrophils and macrophages are the most abundant cell types in the BAL cell pellet, and in control samples ALOX15B mRNA abundance correlates positively, strongly and significantly with macrophage percentage composition of the pellet ($r=0.82$, $p=0.0003$) and negatively with neutrophil percentage composition ($r= -0.82$, $p=0.0003$) (fig. 5c and d). In CF samples, despite variability in the macrophage composition of the samples, the correlation of ALOX15B abundance with macrophage/neutrophil composition was absent ($p=0.20$ and $p=0.17$, respectively) (fig. 5e and f). Variability in the abundance of ALOX5 mRNA, recognised to be expressed abundantly both by neutrophils and alveolar macrophages [26–28], was not significantly correlated with cell type for control or CF samples.

Discussion

This is the first study to demonstrate defective eicosanoid class switching, a key step in initiating the active resolution of inflammation, in the lower airways of young children with CF despite the absence of recognised pathogens. We present *in vivo* evidence of impoverished 15-LO2 gene expression associated with a depressed LXA_4/LTB_4 ratio in the lower airways of children with CF. We found that the correlations between LXA_4/LTB_4 ratio, infection, abundance of 15-LO2 transcripts and macrophages observed in control BAL were lost in CF BAL. This report provides new and important mechanistic insights into the failure to resolve inflammation in CF lung disease.

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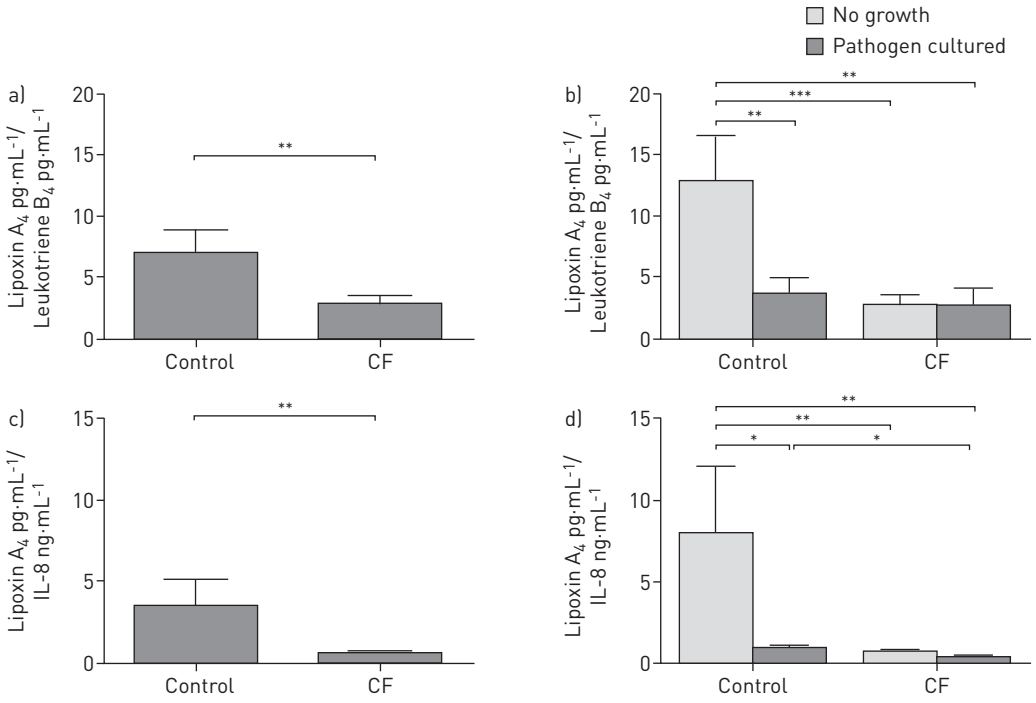

FIGURE 3 Lipoxin A_4 relative to leukotriene B_4 and interleukin (IL)-8. a) Mean lipoxin A_4/leukotriene B_4 ratio measured in control and cystic fibrosis (CF) bronchoalveolar lavage (BAL). b) Mean lipoxin A_4/leukotriene B_4 ratio compared between no growth and pathogen cultured subgroups of the control and CF cohorts. c) Mean lipoxin A_4/IL-8 ratio measured in control and CF BAL. d) Mean lipoxin A_4/IL-8 ratio compared between no growth and pathogen cultured subgroups of the control and CF cohorts. Growth was defined as $>10^4$ CFU·mL^{-1} pathogenic bacteria cultured from the BAL specimen. Error bars represent SEM. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$, t-test.

In order to test the hypothesis that eicosanoid class switching is defective in CF, giving rise to a failure to resolve acute inflammation, we compared eicosanoids temporally associated with the "switch" from propagation to resolution, LTB_4 and LXA_4, respectively. In addition, with the expectation that the phase of inflammation would be affected by the infectious context [29], we conducted subgroup analysis based on the presence or absence of microbial pathogens detected in the BAL fluid. By comparing relative mediator concentrations, expressed as a LXA_4/LTB_4 ratio, we demonstrate that the balance in eicosanoid biosynthesis weighs heavily in favour of LXA_4 production over LTB_4 production in control BAL when compared to CF BAL (fig. 3a). This finding indicates an abnormality in the active resolution of inflammation in CF and is consistent with the reduced LXA_4/IL-8 ratio in CF BAL compared to control BAL also reported here (fig. 3c).

Consistent with a previous report [18], we did not find significant differences when comparing control and CF BAL, directly or in subgroup analyses (based on microbial culture result), as regards the absolute content of LXA_4 (fig. 2a and b). One other study suggested there is a failure to resolve acute inflammation in CF and reported a significant suppression in the ratio of LXA_4/neutrophils in CF [14]. In our study, we did not find any significant difference in the LXA_4/neutrophils ratio between control and CF BAL (online supplementary fig. E1a). This could potentially be explained by differences in the control population between that study and ours, especially with regards to allergic airways disease and infection. Although LTB_4 concentration did not significantly differ between control and CF BAL (fig. 2c), we found that LTB_4 concentration was always higher in infected BAL subgroups (fig. 2d) consistent with its role in neutrophil recruitment and host defence. It is noteworthy that a negative correlation has been described between LTB_4 and pulmonary function outcomes in CF [4].

LXA_4 and its synthetic analogues have been shown, *in vitro*, to suppress IL-8 production by leukocytes and bronchial epithelial cells [11, 12]. As such, the LXA_4/IL-8 ratio represents a compound outcome incorporating both the local concentration of each mediator, and the effectiveness of LXA_4 in antagonising IL-8 production. In control samples the LXA_4/IL-8 ratio was significantly lower in the presence of infection in BAL, consistent with an appropriate pro-inflammatory response to infection. In contrast, the higher LXA_4/IL-8 ratio seen in uninfected control BAL, favouring of the resolution of inflammation, was absent in uninfected CF BAL where the ratio remained low independent of infection.

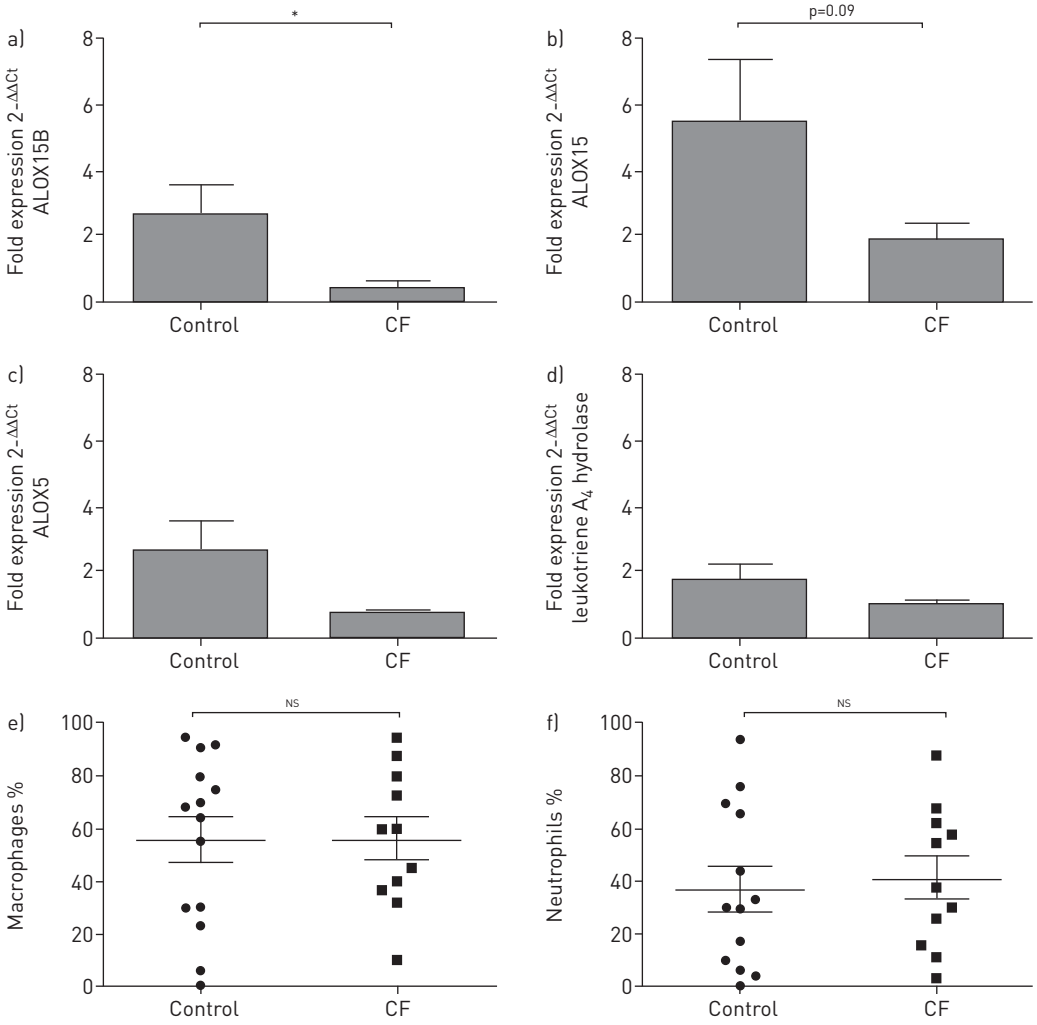

FIGURE 4 Abundance of transcripts for enzymes involved in eicosanoid synthesis quantified in the cellular phase of bronchoalveolar lavage (BAL). Relative abundances of a) arachidonate 15-lipoxygenase type B (ALOX15B), b) arachidonate 15-lipoxygenase (ALOX15), c) arachidonate 5-lipoxygenase (ALOX5) and d) leukotriene A_4 hydrolase mRNA transcripts in the cellular component of the BAL fluid from a subset of the control and cystic fibrosis (CF) samples. Results are expressed as fold expression = $2^{(-\Delta\Delta Ct)}$ compared to a reference patient sample. e) Macrophage and f) neutrophil composition of the control and CF cell pellets expressed as percentage of nucleated cells, as assessed by light microscopy. Error bars represent SEM. NS: nonsignificant. *: $p<0.05$, t-test.

In the absence of airway infection in control children (control samples with negative microbial culture), the balance between LXA_4 and LTB_4 biosynthesis was skewed in favour of LXA_4 production, reflecting ongoing active resolution of inflammation. Furthermore, our study provides *in vivo* evidence that LXA_4/LTB_4 balance in the lower airways of control children correlates strongly with the abundance of 15-LO2 transcripts in the cellular phase of BAL. This finding is consistent with *in vitro* reports that 15-LO enzyme activity promotes LXA_4 biosynthesis at the expense of leukotriene biosynthesis [1, 24]. In the control population, we also find a positive correlation between 15-LO2 transcript abundance and the percentage macrophage composition of the BAL cell pellet, and, reciprocally, a negative correlation with the percentage neutrophils (fig. 4e and f). This is consistent with the recognised expression of 15-LO, and 15(S)-HETE and LXA_4 generation by human alveolar macrophages [21]. Although neutrophils and macrophages in induced sputum both expressed 15-LO, the majority has been attributed to macrophages [30].

Taken together our observations suggest that macrophages, by expressing 15-LO2 and participating in transcellular cooperation with neutrophils expressing 5-LO, make a significant contribution to LXA_4 synthesis in the bronchial lumen and regulate eicosanoid balance *in vivo* in the airway.

In CF, in contrast to our findings in BAL samples from controls, the bias in favour of LXA_4 production in uninfected BAL was absent. LXA_4/LTB_4 and LXA_4/IL-8 ratios were not significantly different between not

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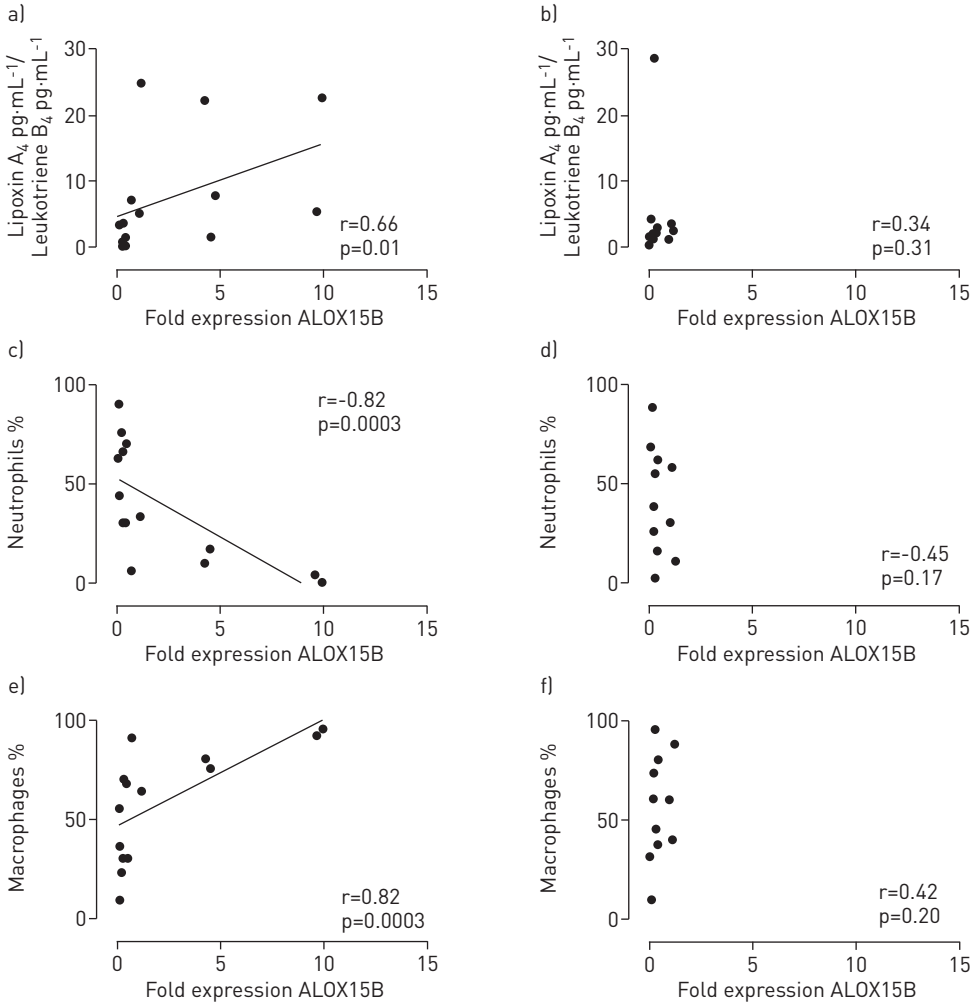

FIGURE 5 Correlation between arachidonate 15-lipoxygenase type B (ALOX15B) transcript relative abundance and lipoxin A_4/leukotriene B_4 ratio from a) control and b) cystic fibrosis (CF) samples. Correlation between ALOX15B transcript relative abundance and percentage neutrophil composition of bronchoalveolar lavage (BAL) from c) control and d) CF samples; and between ALOX15B transcript relative abundance and percentage macrophage composition of BAL from e) control and f) CF samples. ALOX15B mRNA fold expression = $2^{(-\Delta\Delta Ct)}$ compared to a reference patient sample. Percentages of macrophages and neutrophils represent the percentage of nucleated cells in the cellular phase of the BAL fluid assessed by light microscopy. Lipoxin A_4 (pg·mL^{-1})/leukotriene B_4 (pg·mL^{-1}) ratio was measured in the BAL supernatant. The Spearman correlation coefficient (r) and the associated p-value are given for each analysis.

infected and infected CF BAL subgroups (fig. 3b and d). In order to further our understanding of impaired eicosanoid class switching in CF we considered the roles played by various enzymes involved in eicosanoid biosynthesis (12-LO, LTA_4H, 5-LO, 15-LO1 and 15-LO2). The activity of 12-LO in platelets from CF patients has been reported to be reduced [31]; however, in our study, 12-LO transcripts were detected at very low copy numbers in only 10 out of 25 airway samples tested, a finding which could possibly represent blood cell contamination from a friable and inflamed airway wall. Overexpression of LTA_4H could result in a diversion phenomenon, routing the common immediate leukotriene A_4 away from LXA_4 synthesis towards LTB_4 synthesis giving rise to the reduced LXA_4/LTB_4 ratio observed; however, expression was not significantly different in CF BAL than in control BAL. We observed nonsignificant trends towards lower abundance of 15-LO1 and 5-LO transcripts in the CF airway (fig. 4b and c).

Importantly, we found reduced 15-LO2 gene expression in CF samples (fig. 4a). Furthermore, in BAL from children with CF there was a breakdown in the relationships between 15-LO2 transcript abundance, LXA_4/LTB_4 ratio and percentage macrophage composition of the BAL. The macrophage and neutrophil content of the CF BAL cell pellets analysed had a wide range of composition and a similar distribution to the control samples, thus, reduced expression of 15-LO2 is not attributable to the neutrophil cell population simply overwhelming the macrophage population numerically. In fact, reduced 15-LO activity in the face of

preserved 5-LO activity provides a reasonable explanation for the excess of LTB_4 over LXA_4 biosynthesis seen in CF subjects free from infection in this study.

These findings, describing impoverished ability to restore normal eicosanoid mediator balance provide mechanistic insights into the persistence of neutrophil mediated inflammation in lieu of its active resolution in CF lung disease. In light of reports that LXA_4/LTB_4 balance is disturbed across the spectrum of asthma severity [32–35] and in scleroderma lung disease [36], and given that the D-series resolvins [37], protectin D1 [38] and 15(S) HETE (an agonist of the anti-inflammatory nuclear receptor peroxisome proliferator-activated receptor-γ) [39] are also 15-LO products, these mechanistic insights may have wider relevance in the pathophysiology of inflammatory respiratory disease.

15-LO expression is a hallmark feature of the alternative activation phenotype (M2) in macrophages [40], induced by the action of IL-4 and IL-13 (produced by T-helper 2 cells) [41, 42]. While recent studies have identified a bronchial mucosal lymphocytic infiltrate in the CF airway their role in CF airway disease is poorly understood [43]. Deficient expression of IL-4/IL-13, delayed or impaired macrophage differentiation (such as that seen in bronchial epithelial cells in CF [44]), or polarisation towards classical activation (M1) over alternative activation could all contribute to the observed reduction in the expression of 15-LO reported here. In an example of feed-forward amplification, the 15-LO product resolvin D1 promotes M2 macrophage differentiation and, thus, 15-LO deficiency could lead to reduced resolvin D1 biosynthesis and consequently to reduced M2 macrophage differentiation, further compounding the deficiency in 15-LO expression [45]. Alveolar macrophage defects are widely reported in CF, although M1/M2 balance in CF alveolar macrophages remains a subject of contention (reviewed in [46]). Hostile environmental factors could play a role in the failure to actively resolve inflammation observed here. While we examined the impact of conventional microbial infection upon eicosanoid balance we cannot exclude the possibility that sterile inflammatory stimuli (*e.g.* pulmonary aspiration [47] or adherent mucus plaques) or non-culturable microbes [48] affected eicosanoid expression and synthesis. Finally, CFTR expression has been demonstrated in non-CF macrophages and CFTR inhibition disrupted macrophage function; thus, it is plausible that CFTR deficiency could directly affect the regulation of ALOX15B expression, for example *via* endoplasmic reticulum stress and the unfolded protein response [49].

Strengths and limitations

Although there were differences between the mean ages of children with infection in their BAL within the control and CF subgroups, we found no significant correlation between LXA_4/LTB_4 ratio and age among control children ($p=0.31$) or children with CF ($p=0.49$). The measured neutrophil count in our CF cohort is consistently lower than that recently reported for another large study of early lung disease in CF, the AREST CF (Australian Respiratory Early Surveillance Team for Cystic Fibrosis) cohort [50]. There are differences in lavage sampling protocol between the two studies. In our study lavage fluid is obtained from two instillations into the right middle lobe and two into the lingula, with the entire lavage then being pooled and analysed as a homogenous fluid. In the AREST CF study the right middle lobe is lavaged three times, followed by either the lingula or the "most affected lobe on computed tomography (CT)" and different lavage fractions are used for microbiology and inflammation studies [50]. These protocol differences may give rise both to dilutional variation and differences arising from the selection bias introduced by the selection of lavage zone by CT in the AREST CF study. In our study the proportion of children, stratified by age, with infection cultured from BAL was lower than that reported for the AREST CF cohort at similar ages, 14.2% and 33.3% of children in the second and third years of life *versus* 21.1% and 40.2% at the same ages in the AREST CF cohort [50]. The definition of infection we have employed differs from that used by AREST CF, with a more stringent criterion of 10^4 CFU·mL^{-1} *versus* 10^5 CFU·mL^{-1} for positive culture of pathogenic bacteria, whereas the AREST study classified "any" *P. aeruginosa* cultured as infection. The overall prevalence of *P. aeruginosa* was low in this cohort with two children with CF culturing $>10^4$ CFU·mL^{-1} and only one further child with CF culturing "any" *P. aeruginosa* at 900 CFU·mL^{-1}. Repeat analysis was performed classifying "any" *P. aeruginosa* culture as "infection", but did not significantly affect any outcomes reported. Whilst the ELISA method has good sensitivity and specificity, and is widely accepted as a method to measure lipid mediators, it is acknowledged that the mass spectrometry method yields better specificity.

Conclusions

This study provides *in vivo* evidence of impoverished 15-LO2 gene expression associated with a depressed LXA_4/LTB_4 ratio in the lower airways of children with CF. We report new mechanistic insights into the failure to resolve inflammation in early CF lung disease, even in the absence of infection.

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