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Original Article

Airway hyperresponsiveness in FVB/N delta F508 cystic fibrosis transmembrane conductance regulator mice☆

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Abstract

Background: Airway hyperresponsiveness is a feature of clinical CF lung disease. In this study, we investigated whether the FVB/N ΔF508 CFTR mouse model has altered airway mechanics.
Methods: Mechanics were measured in 12–14 week old FVB/N *Cftr*^{tm1Eur} (ΔF508) mice and wildtype littermates using the FlexiVent small animal ventilator. Lung disease was assayed by immunohistochemistry, histology and bronchoalveolar lavage analysis.
Results: *Cftr*^{tm1Eur} mice presented with increased airway resistance, compared to wildtype littermates, in response to methacholine challenge. No differences in bronchoalveolar cell number or differential, or in tissue lymphocyte, goblet cell or smooth muscle actin levels were evident in mice grouped by *Cftr* genotype. The bronchoalveolar lavage of *Cftr*^{tm1Eur} mice included significantly increased levels of interleukin 12(p40) and CXCL1 compared to controls.
Conclusion: We conclude that the pulmonary phenotype of *Cftr*^{tm1Eur} mice includes airway hyperresponsiveness in the absence of overt lung inflammation or airway remodeling.

Keywords: Lung; Airway hyperresponsiveness; *Cftr*; Murine models

1. Introduction

Cystic Fibrosis (CF) patients exhibit a complex lung phenotype that, in addition to infection and inflammation, features structural and mechanistic alterations in the airways and lung tissue [1,2]. CF patients often wheeze and have asthma-like symptoms with airway hyperresponsiveness (AHR) being a component of this phenotype [2]. AHR is the exaggerated response to stimuli within the airways, which has been reported in at least 40% of patients with CF [3], and has been correlated with decreased lung function in CF patients [4].

AHR is a hallmark of asthma and extensive investigations have revealed lung inflammation and airway remodeling to be the major mechanisms leading to this phenotype [5,6]. Specific components of inflammation including T helper cell subsets, T regulatory cells, eosinophils, mast cells, and mediators produced by these cells, have all been linked to AHR in asthma [5]. Airway remodeling can also result in AHR in asthmatics and these structural changes include basement membrane deposition of collagen, airway smooth muscle mass increases, increased vascularity and changes to the extracellular matrix composition [5,6]. A further contributing mechanism may be increased mucous in airways caused by goblet cell hyperplasia [7]. Components of inflammation and airway remodeling have also been linked to AHR in non-asthmatic models including obesity [8] and chronic obstructive pulmonary disease [9].

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Multiple mouse models have been used to investigate the complex CF lung phenotype and although these models present with the altered electrophysiology expected with *Cftr* deficiencies, they do not produce the pulmonary infection phenotype present in CF patients [10]. Mouse models, therefore, allow for investigation of pulmonary mechanics in the absence of infection. Indeed, Cohen et al. [11] have shown that *Cftr*^{tm1UNC} mice, a knockout model, have increased resistance and decreased compliance in lung tissue assayed at baseline while Darrah et al. [12] reported that *Cftr*^{tm1kth} mice, a ΔF508 mouse model, have an increased respiratory rate, each compared to wildtype littermates. Finally, we previously investigated airway mechanics in *Cftr*^{tm1UNC} mice and we identified BALB/c *Cftr*^{tm1UNC} mice to present AHR relative to levels in wildtype mice [13].

To assay mice with a more clinically common mutation in CFTR, in this study we investigated whether altered lung mechanics are a feature of *Cftr*^{tm1Eur} (ΔF508-*Cftr*) mice. The *Cftr*^{tm1Eur} mice were created on a 129/Ola × FVB/N background [14] and subsequently backcrossed to the FVB/N background therefore we were able to evaluate the effects of the *Cftr* mutation, and not of mouse strain, on lung mechanics. Components of this study have been previously published in an abstract [15].

2. Materials and methods

2.1. Mice

+/*Cftr*^{tm1Eur} mice, which had been generated on a 129/Ola × FVB/N background [14] and subsequently backcrossed to the FVB/N background for 12 generations, were bred to generate Cftr +/+ (wildtype) and Cftr −/− (*Cftr*^{tm1Eur}) mice for these studies. *Cftr* genotyping was completed using a previously reported PCR assay [16] with DNA extracted from tail tissue. Due to the risk of death from intestinal obstruction, all mice were given PegLyte (17.8 mmol/L polyethylene glycol, Pharma Science) in the drinking water from weaning at 21 days of age until euthanasia at the experimental endpoint [16,17]. Mice were housed in microisolator cages in specific pathogen free rooms of the animal facility at the Meakins-Christie Laboratories of McGill University. By routine serology surveillance performed on sentinel mice, no viral, mycoplasmal, fungal, or other respiratory pathogens were revealed. Mice were handled according to guidelines and regulations of the Canadian Council on Animal Care.

2.2. Lung function

At 12 to 14 weeks of age, mice were anesthetized using intraperitoneal injections of xylazine (11.3 mg/kg) and pentobarbital (37 mg/kg). To cause diaphragm paralysis, pancuronium bromide (0.2 mg) was injected intraperitoneally. The trachea was exposed and a cannula was inserted before being connected to a computer-controlled ventilator (FlexiVent; SCIREQ®) set at the ventilatory parameters with a respiratory rate of 150 breaths/min, a tidal volume of 10 ml/kg and a positive end-expiratory pressure (PEEP) of approximately 3.0 cmH_2O. Resistance and elastance measurements were recorded, using the forced oscillation technique, after each of the aerosol administration of saline (baseline), and after doubling doses of methacholine (6.25–200 mg/mL) were administered with the Aeroneb ultrasonic nebulizer (SCIREQ®) on the FlexiVent system as in [13]. Mice were subsequently euthanized by cardiac puncture and tissues harvested.

2.3. Histology

The left lung was fixed with 10% neutral buffered formalin and embedded in paraffin. As previously described, slides were stained with hematoxylin and eosin to indicate alveolitis [18] and periodic acid Schiff's stain for goblet cell visualization [13]. All scoring was completed by a user blinded to genotype.

2.4. Immunohistochemistry

Smooth muscle actin immunohistochemistry was performed as previously described [13] using anti-Actin, α smooth muscle antibody (dilution 1:1000, Clone 1A4, Sigma Aldrich). The area of smooth muscle actin was traced using image analysis software (Olympus BX51, Image-Pro Plus 5.1, Media Cybernetics) and normalized for airway size by dividing by the square of basement membrane perimeter (P_{BM}). CD3 immunohistochemistry was completed as previously described [13] using rat anti-human CD3 antibody (dilution 1:75; Serotec; clone CD3-12). CD3+ cells were visualized using avidin–biotin complex–alkaline phosphatase (Vector Laboratories) and then developed with a Vector-red alkaline phosphatase kit (Vector Laboratories). Sections were counterstained with methyl green. The lymphocyte count for each mouse was the number of positive cell counts in ten randomly selected complete lung fields per mouse.

2.5. Bronchoalveolar lavage fluid (BAL) analysis

At necropsy, lavage was performed by cannulating the trachea and retrieving cells from 1-mL injections of phosphate buffered saline augmented with 1% bovine serum albumin. BAL cell differentials were completed as previously described [13] and cytokine levels in BAL supernatants were determined using a Bio-Plex Pro™ Mouse Cytokine 23-plex assay according to the supplier's protocol (Bio-Rad). Interleukin-1 α (IL-1α), IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-17A, eotaxin, ganulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), interferon gamma (IFN-γ), chemokine (C-X-C motif) ligand 1 (CXCL1), monocyte chemotactic protein 1 (MCP-1), macrophage inflammatory protein 1 alpha (MIP-1α), MIP-1β, chemokine (C-C motif) ligand 5 (RANTES), and tumor necrosis factor alpha (TNF-α) were assayed.

2.6. Data analysis

Airway hyperresponsiveness to methacholine was analyzed for mice grouped by genotype using a repeated measure ANOVA followed by a Bonferroni post-test. For all other

group comparisons, Student's *t*-tests were used. A *P* value of less than 0.05 was considered significant.

3. Results

3.1. Airway hyperresponsiveness in *Cftr*^{tm1Eur} mice

A population of 12–14 week old FVB/N mice was produced and the *Cftr*^{tm1Eur} mice were of similar weight to their wildtype littermates (24.5 g ± 3.5 vs. 27.2 g ± 4.0, $P = 0.08$) in agreement with a previous report on this strain [16]. To investigate whether the ΔF508-*Cftr* mutation affects the baseline airway mechanics of FVB/N mice measurements were taken after administration of aeronebulized saline. Analysis with the forced oscillation technique revealed a significant increase in resistance ($P = 0.027$) and elastance ($P = 0.042$) in *Cftr*^{tm1Eur} mice compared to levels in wildtype mice (Fig. 1). To investigate the response of the airways to stimulation, mice were administered increasing doses of methacholine. With this challenge, the *Cftr*^{tm1Eur} mice exhibited an airway hyperresponsiveness indicated by resistance measures exceeding those of wildtype mice, as shown in Fig. 1 ($P = 0.047$). The elastance measures recorded in response to increasing doses of methacholine did not significantly differ between *Cftr*^{tm1Eur} and wildtype mice ($P = 0.15$), although at the dose point of 200 mg/ml of methacholine elastance in *Cftr*^{tm1Eur} mice significantly exceeded levels in wildtype mice ($P < 0.01$; Fig. 1).

3.2. Airway remodeling in *Cftr*^{tm1Eur} mice

To identify whether components of airway remodeling are altered in the lungs of *Cftr*^{tm1Eur} mice presenting hyperresponsiveness, we assayed goblet cell counts and α-smooth muscle actin (SMA) area in lung tissue. In contrast to their increased presence in asthmatic mouse models [7], goblet cells were very rare in the lungs of each of *Cftr*^{tm1Eur} and wildtype mice, with most airway sections having no goblet cells (Fig. 2A and B). Secondly, no significant increase in SMA area in any of large, medium or small airways was evident in the FVB/N *Cftr*^{tm1Eur} mice (Fig. 2E; $P > 0.23$).

3.3. Lung inflammation in *Cftr*^{tm1Eur} mice

In addition to airway remodeling, inflammation has been reported to contribute to the development of AHR in asthma [5,6]. To investigate whether the lung tissue of *Cftr*^{tm1Eur} mice was inflamed, we used histology to score alveolitis and to identify lymphocytes. No difference in the amount of alveolitis, based on semi quantitative scoring of the lung tissues, was evident between *Cftr*^{tm1Eur} and wildtype mice (Fig. 3A to C). Secondly, CD3+ cell counts did not differ in mice grouped by *Cftr* genotype as shown in Fig. 3D to F.

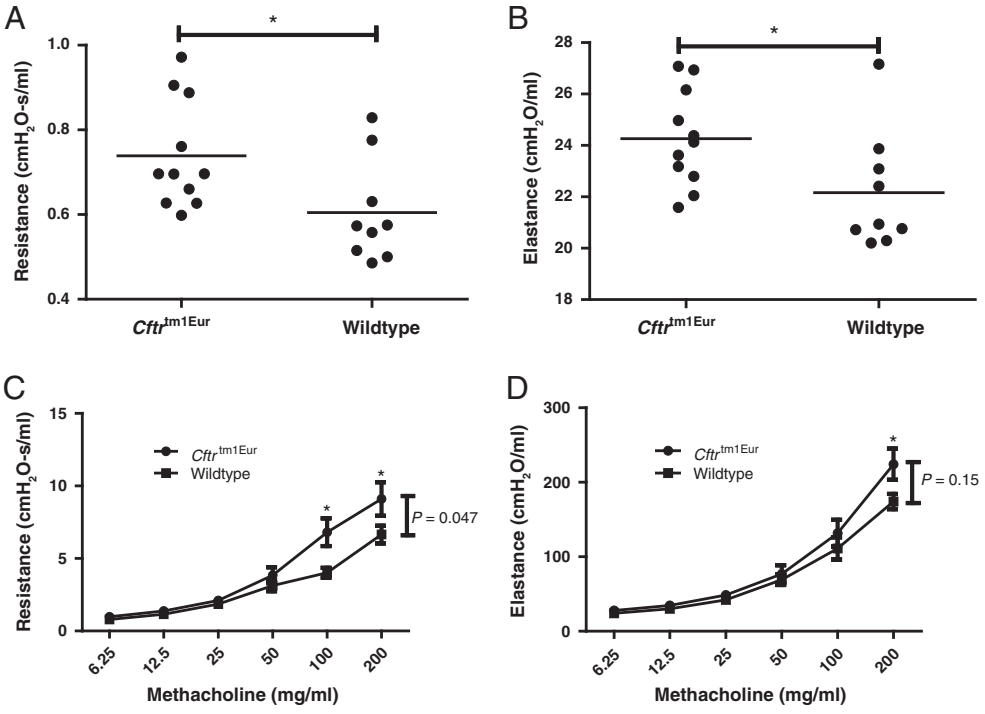

Fig. 1. Respiratory mechanics of FVB/N wildtype and *Cftr*^{tm1Eur} mice at baseline and after methacholine challenge. Tracheostimized 12–14 week old mice received saline (baseline) and increasing doses of aeronebulized methacholine and mechanics were assayed on a FlexiVent instrument. (A) Resistance and (B) elastance measurements were obtained using the forced oscillation technique at baseline. Horizontal line indicates mean value. (C) Resistance and (D) elastance measurements were obtained for increasing doses of methacholine. Results are from a single experiment with 9 to 11 mice per group; mean ± standard error. Vertical bar indicates a significant difference between wildtype and *Cftr*^{tm1Eur} mice as determined by repeated measures ANOVA. *Significant difference between wildtype and *Cftr*^{tm1Eur} mice.

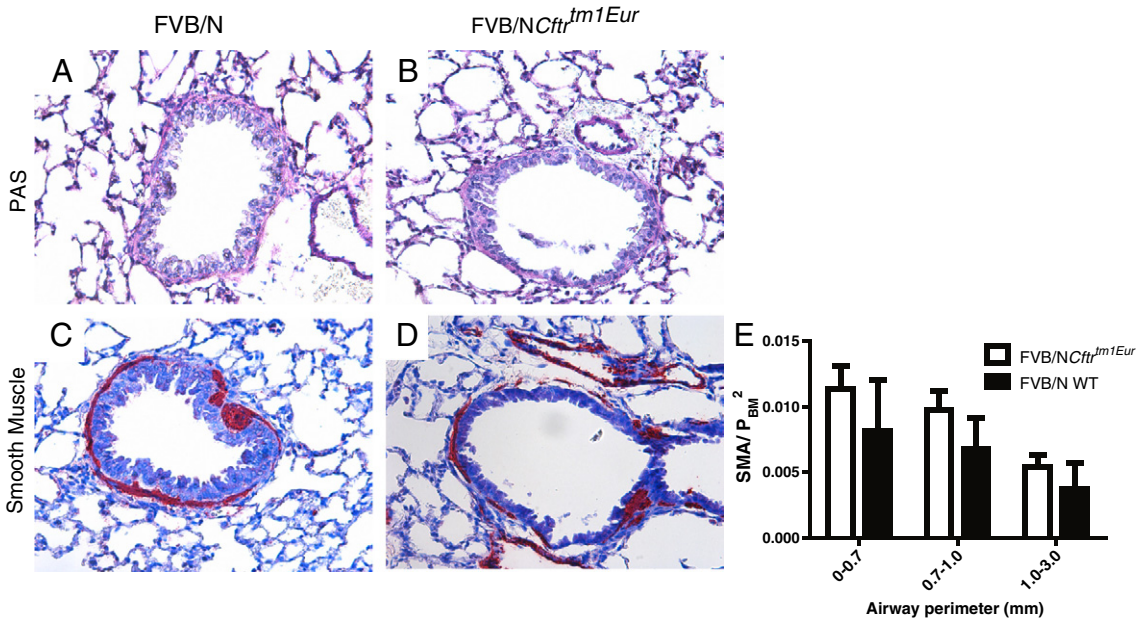

Fig. 2. Goblet cells and alpha smooth muscle actin in lung tissue of FVB/N wildtype and *Cftr*^{tm1Eur} mice. Lungs were procured from 12 to 14 week old mice and histologically processed. Periodic acid Schiff staining to indicate mucous and goblet cells staining of (A) FVB/N wildtype and (B) FVB/N *Cftr*^{tm1Eur} mice; magnification = 400×. α-Smooth muscle actin (SMA) immunohistochemical staining of (C) FVB/N wildtype and (D) FVB/N *Cftr*^{tm1Eur} mice; magnification = 400×. (E) SMA area per basement membrane perimeter squared, shown at three different airway sizes. Results are from a single experiment with 3 to 6 mice per group. Values are mean ± standard deviation. No significant difference between wildtype and *Cftr*^{tm1Eur} mice, in SMA area, was determined for any airway size $P > 0.23$.

3.4. Cell differential and cytokines in bronchoalveolar lavage in Cftr^{tm1Eur} mice

Further to tissue inflammation we investigated the bronchoalveolar lavage (BAL) cell numbers, cell differential and cytokine levels. The total BAL cell number did not differ between *Cftr*^{tm1Eur} and wildtype mice ($9.8 \times 10^3 \pm 6.8 \times 10^3$ vs. $6.25 \times 10^3 \pm 2.5 \times 10^3$, $P = 0.14$) and eosinophils were not detected in either group. As shown in Fig. 4A, the cellular differential in the BAL did not differ between *Cftr*^{tm1Eur} and

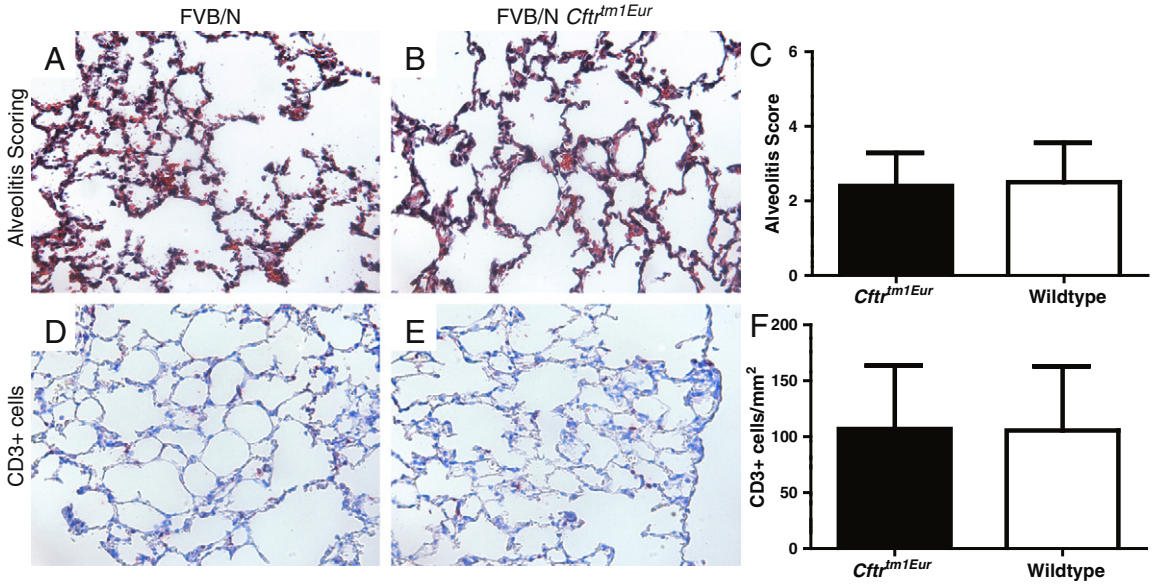

Fig. 3. Inflammatory markers in lung tissue of FVB/N wildtype and *Cftr*^{tm1Eur} mice. Lungs were procured from 12 to 14 week old mice and histologically processed. (A) FVB/N wildtype and (B) FVB/N *Cftr*^{tm1Eur} mouse lung tissue stained with hematoxylin and eosin; magnification = 400×. (C) Alveolitis score by semi quantitative scoring of hematoxylin and eosin stained lung tissue; average score ± SD, n = 5 mice per group. CD3 immunohistochemistry of (D) FVB/N wildtype and (E) FVB/N *Cftr*^{tm1Eur} lung tissue; magnification = 400× (F) Average CD3 positive cells ± SD per mm^2 of lung tissue from counts of histological sections. Results are from a single experiment with 10–11 mice per group. No significant difference in alveolitis score, PMN count or CD3+ count in mice grouped by *Cftr* genotype $P > 0.65$.

wildtype mice. We next assessed the levels of cytokines in the BAL to determine whether the AHR in *Cftr*^{tm1Eur} mice was associated with differential cytokine levels. As seen in Fig. 4B, lavage from FVB/N *Cftr*^{tm1Eur} mice had increased levels of Il-12(p40) and chemokine (C-X-C motif) ligand 1 (CXCL1) compared to wildtype mice, while levels of TNFα did not differ between the groups. The remaining cytokines were not expressed at levels beyond the detection limit of the assay.

4. Discussion

We have shown *Cftr*^{tm1Eur} mice, which have the clinically relevant ΔF508 mutation, to have altered airway mechanics and to present with airway hyperresponsiveness to methacholine challenge, when compared to wildtype littermates. *Cftr*^{tm1Eur} mice do not present with increased airway muscle area, goblet cell infiltration or airway/lung tissue inflammatory cell infiltration. Levels of Il-12(p40) and CXCL1 were greater in the lavage supernatant from the *Cftr*^{tm1Eur} mice compared to wildtype controls.

Our results indicate that a lack of functional *Cftr* changed airway mechanics, both in unstimulated and methacholine stimulated lungs, in agreement with prior studies of CF mouse models. In detail, we have shown previously that in a *Cftr* knockout mouse model there is a strain dependent AHR phenotype which is present in BALB/c *Cftr*^{tm1UNC} mice, but not C57BL/6 *Cftr*^{tm1UNC} mice, as measured by increased resistance to methacholine. In agreement with the airway hyper-reactive BALB/c *Cftr*^{tm1UNC} mice, the FVB/N *Cftr*^{tm1Eur} mice of the current report did not present with increased airway smooth muscle area or goblet cell numbers [13]. The AHR in BALB/c *Cftr*^{tm1UNC} mice was, however, associated with higher levels of CD3 + cells in the lung tissue of these mice [13]; and this phenotype was not evident in the FVB/N *Cftr*^{tm1Eur} mice. Groups using other analysis techniques have also shown altered airway mechanics to exist in CF. For example, Darrah et al. [12] used whole-body plethysmography to assay the response of a different ΔF508 mouse model (*Cftr*^{tm1kth}), on a C57BL/6 background, and showed these mice to have an increased breathing rate compared to wildtype, in agreement with studies on CF patients [19]. Cohen et al. [11] employed the constant phase model on data from *Cftr*^{tm1UNC} mice, also on the C57BL/6 background, to show that unstimulated CF lungs had increased Newtonian resistance, tissue dampening and tissue elastance when compared to wildtype lungs, thus supporting the observation in FVB/N *Cftr*^{tm1Eur} mice of an effect of the *Cftr* mutation on baseline airway mechanics.

AHR has been documented in CF patients but the underlying physiological mechanism differs from that of asthma based on data from both patients and mouse models. AHR is a hallmark of asthma and in CF patients there is a possibility of concomitant asthma [1]. However, AHR in CF patients is generally different from AHR in asthmatic patients. For example, Mitchell et al. showed that 51% of CF patients showed AHR to methacholine challenge, but less than half of these patients developed AHR in response to a histamine challenge [20]. In contrast, asthmatic patients have high concordance between methacholine and histamine induced AHR [2]. Further, CF patients generally do not develop AHR in response to exercise [21], which is commonly seen in asthmatic patients [6]. In animal models the development of asthma is usually associated with increases in both resistance and elastance in response to methacholine [22,23] while results from the current study of the lung phenotype of FVB/N *Cftr*^{tm1Eur} mice indicate, in contrast, increased resistance measurements following methacholine challenge, but no significant increase in elastance measures.

The mechanisms which lead to AHR in CF have not been elucidated but may involve altered smooth muscle function or may be affected by pulmonary inflammation, phenotypes commonly seen in CF patients [24]. Clinical studies have shown that both CF children [25] and adults [26,27] have increased airway smooth muscle mass compared to levels in healthy controls, but as this phenotype was not evident in the FVB/N *Cftr*^{tm1Eur} mice, the hyperresponsiveness in the animal model cannot be attributed to this trait. However, given that airway smooth muscle expresses CFTR [28], it is possible that the reduced function of CFTR in the airway smooth muscle could alter its properties and thus contribute to the AHR phenotype. Supporting this assertion are the findings of Michoud et al. [28]

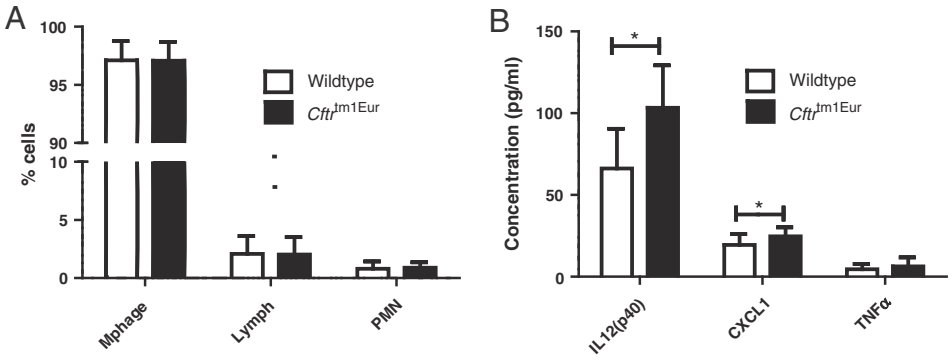

Fig. 4. Bronchoalveolar lavage cell differentials and cytokine levels of FVB/N wildtype and *Cftr*^{tm1Eur} mice. Lavage was procured from 12 to 14 week old mice. A) Percent of each cell type measured in the bronchoalveolar lavage; Lymph = lymphocyte, PMN = polymorphonuclear leukocytes, Mphages = macrophages. Results are from a single experiment with 4–7 mice per group mean ± SD of groups. No significant differences between groups of mice were evident, $P > 0.6$. B) BAL supernatants were assayed using a 23-plex. Cytokines with values below the detection limit are not shown. Data presented are means ± SD of a single experiment with 13 mice per group. *Indicates significant difference between groups, $P < 0.05$.

who reported airway smooth muscle cells from CF patients to release decreased amounts of Ca^{2+} in response to histamine stimulation, compared to cells from non-CF patients. Pulmonary inflammation has also been linked to AHR in asthma [6], but was limited in our CF model. Specifically we did not detect any tissue level inflammatory cell infiltration, nor changes to the BAL differential or cell count of FVB/N *Cftr*^{tm1Eur} mice. We did, however, identify increased levels of CXCL1 and Il-12(p40) in the lavage of the FVB/N *Cftr*^{tm1Eur} mice which may be mechanistically significant as both IL-12 and CXCL1 levels are known to affect lung mechanics [29,30]. Whether these cytokines, or others if detected by more sensitive assays, directly contribute to the AHR phenotype in CF has not been investigated.

The *Cftr*^{tm1Eur} line is an important mouse model as it carries the clinically common ΔF508 mutation and because extensive resources have been allocated to identifying compounds to treat the trafficking defect associated with the mutation. Our finding of altered airway mechanics in this mouse model allows for investigations into this phenotype, which enhances the preclinical utility of this model. Investigations with this CF mouse model, which presents with altered airway mechanics but no airway remodeling or overt inflammatory cell infiltration, permit insights into the mechanism leading to this CF phenotype to be gained.

Conflict of interest statement

The authors have no conflicts of interest to declare.

Acknowledgments

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