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Airway remodelling and inflammation in asthma are dependent on the extracellular matrix protein fibulin-1c

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Abstract

Asthma is a chronic inflammatory disease of the airways. It is characterised by allergic airway inflammation, remodelling and hyperresponsiveness (AHR). Asthma patients, in particular those with chronic or severe asthma, have airway remodelling that is associated with the accumulation of extracellular matrix (ECM) proteins, such as collagens. Fibulin-1 (Fbln1) is an important ECM protein that stabilises collagen and other ECM proteins. Fbln1c, one of the four Fbln1 variants, which predominates in both humans and mice, is increased in the serum and airways fluids in asthma but its function is unclear. We show that Fbln1c protein was increased in the lungs of mice with house dust mite (HDM)-induced chronic allergic airway disease (AAD). Genetic deletion and therapeutic inhibition of Fbln1c in mice with chronic AAD reduced airway collagen deposition, and protected against AHR. Fbln1c deficient (−/−) mice had reduced mucin MUC5AC, but not MUC5B protein levels in the airways compared to wild-type (WT) mice. Fbln1c interacted with fibronectin and periostin that was linked to collagen deposition around the small airways. Fbln1c−/− mice with AAD also had reduced α-smooth muscle actin positive cells around the airways and reduced airway contractility compared to WT mice. These mice also had less airway inflammatory cells, as well as reduced levels of IL-5, IL-13, IL-33, TNF and CXCL1 levels in the lungs, and IL-5, IL-33, and TNF protein in lung-draining lymph nodes after HDM challenge. Therapeutic targeting of Fbln1c reduced the numbers of GATA3+ Th2 cells in lymph nodes and lungs after chronic HDM challenge. Treatment also reduced the secretion of IL-5 and IL-13 protein from co-cultured dendritic cells and T cells re-stimulated with HDM. Human epithelial cells cultured with Fbln1c
peptide produced more \textit{CXCL1} mRNA than medium-treated controls Our data show that Fbln1c may be a therapeutic target in chronic asthma.
Introduction

Asthma is a chronic inflammatory respiratory disease predominantly of the airways. It is characterised by airway inflammation and remodelling that leads to airway hyperresponsiveness (AHR) and reversible airflow obstruction [1,2]. Clinical symptoms include shortness of breath, wheeze, chest tightness and dry cough [3]. There are more than 300 million people suffering from asthma worldwide [4]. Asthma exacerbations are a major problem causing increased symptoms and hospitalisations and are triggered by multiple allergic and exogenous stimuli, such as respiratory infections, house dust mites (HDM), pollen, and occupational chemicals [5,6]. The symptoms of disease can be controlled in mild to moderate allergic asthmatics using combination therapies with inhaled corticosteroids and long acting-β agonists, while new biologics such as omalizumab (anti-IgE antibody), dupilumab (anti-IL-4Rα antibody) and mepolizumab (anti-IL-5) are also showing promise [1,2]. However, these medications only treat the symptoms, while the underlying disease features, in particular chronic airway remodelling, are not altered.

Airway remodelling is an important feature of chronic asthma, which includes excessive extracellular matrix (ECM) production and collagen deposition leading to airway fibrosis, increased airway smooth muscle (ASM) cell mass, mucus hypersecretion and elevated numbers of fibroblasts/myofibroblasts [7-9]. Aberrant deposition of ECM proteins is a hallmark characteristic of chronic asthma that causes airway stiffening and narrowing, and differences in ECM protein expression may represent a specific asthma endotype(s) [10]. There are significant increases in type I and
III collagen gene expression and protein content in the airways in chronic asthma [11,12]. In contrast, type IV collagen deposition is decreased around the airways [13]. Other ECM components, including fibronectin (Fn), tenacin-c (Tnc) and periostin (Postn), have also been shown to occur at aberrant levels in the airways of asthma patients [14-16].

Fibulin-1 (Fbln1) is a secreted glycoprotein found in the ECM in asthma [17]. It facilitates the stabilisation of other ECM proteins, including Fn, Postn and Tnc in the lung [18,19]. There are four variants of Fbln1 (Fbln1a, b, c and d) in humans, with each one expressing a different C-terminal sequence. Fbln1c and d are the predominant variants in adult humans and mice [20]. We have shown that the levels of total Fbln1 protein are increased in the serum and bronchoalveolar lavage fluid (BALF) of asthma patients compared to non-asthmatic subjects [21]. Our in vitro studies have shown that stimulation of ASM cells from asthmatic patients with TGF-β increases the levels of secreted Fbln1 protein [21]. However, the protein levels specifically of Fbln1c in asthma patients are unknown. We also showed that antisense oligonucleotide silencing of Fbln1c reduces ASM cell proliferation [21], and that Fbln1c deficient (−/−) mice have less collagen around the small airways in a mouse model of chronic asthma [22]. However, the in vivo function of Fbln1 in asthma, and in particular its role in airway remodelling and inflammation has not been assessed.

In this study, we demonstrate that Fbln1c is essential for the development and progression of airway remodelling in chronic HDM-induced experimental asthma (allergic airway disease [AAD]). Deletion of Fbln1c in mice prevents the development of airway fibrosis and collagen deposition, as
well as inflammation. This suggests that Fbln1c may be a novel therapeutic
target in the treatment of airway diseases such as asthma by suppressing
chronic disease characteristics, such as airway remodelling and inflammation.

Materials and Methods
Additional details are provided in supplementary materials, Supplementary
materials and methods.

All mouse experiments were approved by the Animal Ethics Committee of The
University of Newcastle.

Mice and HDM-induced experimental chronic asthma
Six to eight-week-old female WT or Fbln1c−/− C57BL/6J mice were housed in
specific pathogen free conditions. Fbln1c−/− mice were generated as described
previously [22]. Experimental chronic asthma was induced by intranasal
administration of HDM extract (Geer Laboratories, Lenoir, NC, USA) at 25 μg
in 30 μl sterile saline for five consecutive days per week for five weeks to
induce the hallmark features of chronic asthma including airway remodelling
as previously described [22,23] (Figure 1A). Control mice received sterile
saline only. Some mice were treated intranasally with 40 μg Fbln1c siRNA or
scrambled siRNA (Dharmacon, Lafayette, CO, USA) [22] or nuclease free
water (vehicle) from day 21 to day 35 after the initiation of airway remodelling.

Airway remodelling and AHR
Sections of formalin-fixed paraffin embedded mouse lung were deparaffinised and collagen was stained using a Sirius Red and Fast Green stain (Sigma-Aldrich, Castle Hill, NSW, Australia). Airway remodelling in terms of collagen deposition around the small airways was analysed using ImageJ (version 1.47, Media Cybernetics, Rockville, MD, USA) [22,24]. AHR was measured as described previously [25-27].

Protein extraction, immunoblotting, hydroxyproline and soluble collagen assays
Lung tissues were homogenised, proteins were detected using antibodies, hydroxyproline content and soluble collagen in whole mouse lungs were assessed as described previously [22,28-33] and in supplementary material, Supplementary materials and methods.

Mucus secreting cells
Mouse lung sections were stained with Periodic acid-Schiff (PAS) and the number of mucus secreting cells around the airways were enumerated [29,34].

Immunostaining and immunofluorescence assays
Mouse lung sections were incubated with antibodies described as previously described [22,28,29] and in supplementary material, Supplementary materials and methods.

Airway contractility
Mouse lungs were inflated, sectioned and exposed to increasing concentrations of methacholine, and airway contractility was assessed as described previously [35,36].

**BALF and ELISA**

BALF was collected, and differential leucocyte counts were determined. ELISA were assessed from mice as described previously [22,27,37,38].

**Lymph node and lung assays**

Mouse lung draining lymph nodes and lungs were collected [39]. The cells were cultured with or without 5 μg/ml HDM for five days, and the levels of secreted IL-5 and IL-13 were measured by ELISA [27,38]. Some cells were stained with Th2 cell markers and enumerated by flow cytometry.

**Dendritic cell (DC) and T cell cytokine release**

DCs and T cells were isolated, co-cultured and the levels of secreted IL-5 and IL-13 were measured by ELISA [39,40].

**In vitro experiments**

Human respiratory epithelial A549 cell line and minimally immortalised epithelium-derived basal (BCi-NS1.1) cells were cultured with Fbln1c peptide and scrambled peptide as previously described [41]; RNA extraction and qPCR were performed as described previously [42,43] in supplementary material, Supplementary materials and methods.
Statistics

Data are presented as mean ± standard error of the mean (SEM) from 4–8 mice, in duplicate or triplicate experiments. Statistical analyses are detailed in supplementary material, Supplementary materials and methods.

Results

Airway remodelling and AHR are associated with increased levels of Fbln1c in experimental chronic asthma

Fbln1c mRNA levels were increased in airway smooth muscle cells derived from asthma patients [21]. In order to determine the role of Fbln1c in airway remodelling in chronic asthma, we employed an experimental model of HDM-induced chronic asthma as described previously [22,23]. Airway remodelling was determined by assessing the levels of collagen deposition around the airways. A substantial (two-fold) increase in collagen deposition around the small airways was observed in mice exposed to HDM for 21 days, and after 35 days compared to saline-challenged controls (Figure 1B). The levels of type I collagen-α1 (Col1a1), the most abundant collagen in the lungs, were increased from 28 to 35 days HDM challenge (Figure 1C). These mice also developed AHR, characterised by increased airway resistance and decreased dynamic compliance in response to increasing concentrations of methacholine, compared to controls after 35 days HDM challenge (Figure 1D). Fbln1 and Fbln1c protein levels were significantly increased in the lung tissue from 28 to 35 days of HDM-exposed mice compared to controls, assessed using immunoblotting (Figure 1, E,F). Thus, these data show that
airway remodelling and AHR are associated with increased levels of Fbln1c in the lungs in HDM-induced experimental chronic asthma.

Genetic deletion of *Fbln1c* protects against airway and lung remodelling and AHR in experimental chronic asthma

In order to assess the role of Fbln1c in airway remodelling and AHR in asthma, *Fbln1c* deficient (+/−) mice were created as we have previously described [22], and chronically exposed to HDM. The absence of *Fbln1c* completely inhibited the deposition of collagen around the small airways compared to WT mice after chronic HDM challenge (Figure 2A), and our previous study showed similar data [22]. Increases in total and soluble collagen in lung tissue (Figure 2B), as well as levels of Col1a1 were completely inhibited (Figure 2C) in *Fbln1c*+/− mice. Indeed, levels of collagen deposition, total and soluble collagen and Col1a1 in *Fbln1c*+/− mice exposed to HDM were equivalent to baseline levels in saline-challenged control WT and *Fbln1c*−/− mice, which were equivalent to each other.

Goblet cell hyperplasia/metaplasia and mucus hypersecretion is also a chronic feature of asthma. Thus, we assessed the impact of the absence of Fbln1c on this feature. Lung sections were stained with PAS, and mucus hypersecretion was assessed by quantifying the number of PAS-positive cells to a depth of 100 μm beneath the epithelial basement membrane. Chronic HDM challenge induced significant increases in the numbers of PAS-positive cells in the airways in both WT and *Fbln1c*−/− mice compared to their controls. However, there was no difference between the number of PAS-positive cells in the airways of HDM-challenged WT or *Fbln1c*−/− mice (see supplementary
material, Figure S1A,B). We also assessed the levels of mucin MUC5AC and MUC5B in the airways using immunofluorescence. *Fbn1c*−/− mice had significantly reduced MUC5AC but not MUC5B protein levels in the airways after HDM challenge (supplementary material, Figure S2).

AHR and wheezing are major disease symptoms of chronic asthma. In order to determine whether pathological changes identified in *Fbn1c*−/− mice affected these functional changes, AHR was assessed in terms of increased airway resistance and decreased dynamic compliance in response to increasing concentrations of methacholine. Increased airway resistance (Figure 2D) and decreased dynamic compliance (Figure 2E) were induced in response to chronic HDM exposure in WT mice but did not develop in *Fbn1c*−/− mice.

We then assessed whether siRNA to suppress levels of Fbln1c could be used therapeutically. Since airway remodelling occurred after 21 days of HDM challenge (Figure 1B), *Fbn1c*-targeted or scrambled siRNA or vehicle were administered every two days from day 21 to day 35 of HDM challenge. *Fbn1c* siRNA treatment after the establishment of remodelling reduced the levels of collagen deposition around the small airways (Figure 2F) and Col1a1 protein (Figure 2G) in the lungs. The use of immunoblotting identifies Col1a1 (125 kDa) from Col1a2 (110 kDa). Treatment also protected against chronic HDM-induced AHR (Figure 2H,I).

These data demonstrate that Fbln1c is necessary for the development of airway remodelling and AHR in HDM-induced experimental chronic asthma.
Fbln1c has varied roles in the accumulation of Fn, Tnc, and Postn around the airways and in the lung in experimental chronic asthma. The increased deposition of ECM proteins underpins airway remodelling, and we showed previously that Fbln1 is critical for ECM stabilisation [22]. Thus, the role of Fbln1c in the deposition of ECM associated proteins in HDM-induced chronic asthma was examined. HDM challenge of WT mice resulted in substantial (two-fold) increases in the protein levels of Fn, Postn and Tnc around the small airways compared to saline-challenged controls (Figure 3A–C). However, HDM-challenged Fbln1c−/− mice had only a partial increase in Fn and were completely protected against increases in Postn. The increase in Tnc occurred to the same levels in HDM-exposed Fbln1c−/− and WT mice.

Next, we assessed the levels of these proteins in the whole lung using immunoblots. HDM challenge of WT mice resulted in increased levels of Fn and Postn in whole mouse lungs, while Tnc levels were reduced (Figure 3D). It should be noted that different isoforms of Tnc can be detected by immunoblot but not IHC in which all forms are detected simultaneously. HDM challenged Fbln1c−/− mice were completely protected against the increases in Fn and Postn that occur in WT mice. The levels of Tnc were reduced in HDM-challenged WT mice compared to saline-challenged controls. However, there was no statistically significant reduction in HDM-challenged Fbln1c−/− mice compared to their controls (P=0.165 for isoform 1, P=0.1097 isoform 2).

These data demonstrate that Fbln1c is necessary for the increased levels of Fn and Postn but not Tnc around the airways and in lung tissue in HDM-induced experimental chronic asthma.
Fbln1c is required for increases in the levels of α-smooth muscle actin (α-SMA) positive cells around the airways and contractility of the airways in experimental chronic asthma.

Increased numbers of α-SMA positive cells is a major feature of airway remodelling in asthma [8,9]. Thus, to further clarify the role of Fbln1c in airway remodelling in asthma, we next assessed the number of α-SMA positive cells around the airways in Fbln1c/− and WT mice using immunofluorescence. Following chronic HDM challenge of WT mice there was a doubling of α-SMA positive cells around the medium (Figure 4A) and small (Figure 4B) airways compared to saline-challenged controls. HDM-challenged Fbln1c/− mice were completely protected against these increases in α-SMA positive cells. Indeed, the number of α-SMA positive cells in Fbln1c/− mice challenged with HDM was the same as the baseline numbers in saline-challenged control WT and Fbln1c/− mice.

Increased ASM results in abnormal airway contractility in asthma. Thus, we then assessed the role of Fbln1c in airway contraction by measuring airway lumen area in response to methacholine in precision cut lung slices [35,36]. Chronic HDM challenge of WT mice resulted in a greater reduction in airway lumen area compared to saline-challenged controls (Figure 4C). HDM-challenged Fbln1c/− mice were protected against this increased contractility, which was equivalent to baseline levels in saline-challenged WT and Fbln1c/− mice (Figure 4D).

These data show that Fbln1c is necessary for the increased levels of α-SMA positive cells around the airways and increased airway contractility in HDM-induced experimental chronic asthma.
Fbln1c contributes to pulmonary inflammation in experimental chronic asthma

Chronic airway inflammation is an important feature and driver of pathogenesis in asthma. Thus, we next assessed if Fbln1c had any role in inflammation in chronic experimental asthma. Total leukocyte numbers as well as macrophages, neutrophils, eosinophils and lymphocytes, were significantly increased in the BALF of WT mice after chronic HDM challenge (Figure 5A). All these cell types were also increased in HDM-challenged Fbln1c−/− mice, however the levels of neutrophils, eosinophils and lymphocytes were significantly less than in HDM-challenged WT mice. HDM challenge of WT mice resulted in significant increases in the levels of the proinflammatory cytokines, IL-5, IL-13, IL-33, TNF and the chemokine CXCL1 in the lung tissue compared to saline-challenged controls (Figure 5 B–F). However, Fbln1c−/− mice were completely protected against HDM-induced increases in these factors, with levels no different to those in saline-challenged WT and Fbln1c−/− mice, which were equivalent. We also enumerated GATA3+ Th2 cells in lymph nodes between naive WT and Fbln1c−/− mice by flow cytometry, and the found that there were no differences (supplementary material, Figure S3).

Since genetic deletion in Fbln1c−/− mice reduced chronic HDM-induced inflammation, the therapeutic effects of Fbln1c-targeted siRNA treatment were assessed. Treatment reduced the levels of total leukocytes and neutrophils, eosinophils, and lymphocytes (Figure 5G) in BALF, and IL-5, IL-13, IL-33, TNF, and CXCL1 proteins (Figure 5 H–L) in the lungs compared to scrambled
siRNA treatment or vehicle after chronic HDM challenge. There were also fewer GATA3+ Th2 cells in lymph nodes (Figure 5M and supplementary material, Figure S3) and lungs (Figure 5N and supplementary material, Figure S4) from mice treated with Fbln1c siRNA.

Fbln1c regulates lymph node T-cell cytokine secretion in experimental chronic asthma

In order to further assess the role of Fbln1c in regulating inflammation in asthma, the lymph nodes of WT and Fbln1c−/− mice challenged with HDM or saline were collected. The secretion of IL-5, IL-13 and TNF were all substantially increased from lymph node T-cells from HDM-challenged WT mice compared to saline-challenged controls (Figure 6,A-C). T-cells from HDM-challenged Fbln1c−/− mice secreted significantly lower levels of IL-5 and IL-13, which were no different to baseline levels in saline-challenged WT and Fbln1c−/− mice, which were equivalent. T-cells from HDM-challenged Fbln1c−/− mice also had lower levels of TNF secretion compared to similarly challenged WT mice, although levels were still elevated compared to saline-challenged Fbln1c−/− controls.

We next determined if Fbln1c affected DC and/or Th2 cell activity and/or DC-induced Th2 responses by measuring the levels of secreted IL-5 and IL-13 protein from co-cultured DC and T cells re-stimulated with HDM. Fbln1c-targeted siRNA treatment affected both DC and Th2 cells and reduced IL-5 (Figure 6D) and IL-13 (Figure 6E) production compared to cells from mice treated with scrambled siRNA. This occurred when either Fbln1c siRNA treated DCs or T cells were cultured with scrambled siRNA treated T cells or
DCs, respectively. The greatest effect was when DCs and T cells were isolated from *Fbln1c* siRNA treated mice and co-cultured. Thus, Fbln1c affects DCs and T cells and promotes DC-induced Th2 responses.

**Fbln1c induces chemokine mRNA expression from a human airway epithelial cell line**

We then undertook studies with human cells to validate some of our findings. Fbln1c recombinant protein is not available, and so human Fbln1c and scrambled peptides were obtained as described previously [42]. Human bronchoepithelial A549 cells and minimally immortalised epithelium-derived basal (BCi-NS1.1) cells were cultured on plates coated with Fbln1c, scrambled peptides, or were uncoated and contained media only, and RNA was extracted after 24 and 48 h. *CXCL1* RNA levels were significantly increased in cells exposed to Fbln1c peptide compared to culture medium and there were non-statistically significant trends towards increases compared to scrambled peptide treatment (P<0.06 and P<0.08, respectively, Figure 6F,G).

**Discussion**

Increased production and deposition of ECM proteins, resultant airway remodelling and hyperresponsiveness, and pulmonary inflammation are key pathological features of asthma. Our previous studies have shown that Fbln1 is increased in BALF and ASM cells from asthma patients compared to healthy controls [21]. In this study, we have demonstrated important roles for Fbln1c in regulating the chronic features of airway remodelling and inflammation in experimental chronic asthma. We found that Fbln1, Fbln1c
and collagen proteins are increased in a HDM-induced mouse model of experimental chronic asthma. Genetic and therapeutic depletion of Fbln1c prevented the increases in chronic HDM-induced collagen around the small airways and in the lungs. It also prevented the development of HDM-induced AHR. Fbln1c−/− mice had fewer α-SMA positive cells around the airways and less airway contractility that likely also contributed to the protection against AHR. We also showed that Fbln1c is involved in allergic airway inflammation, because depletion of Fbln1c in mice resulted in fewer leukocytes, including neutrophils, eosinophils and lymphocytes, which had influxed into the airways compared to controls. The levels of proinflammatory cytokines and chemokines associated with allergic airway inflammation and the influx of neutrophils, eosinophils and Th2 lymphocytes into the lungs were also reduced. Fbln1c−/− mice also had reduced levels of IL-5, IL-13 and TNF secretion by Th2 cells from lung draining lymph nodes compared to WT mice. Depletion of Fbln1c also reduced IL-5 and IL-13 secretion from co-cultured DC and T cells after HDM stimulation. In support of the increase in inflammatory responses induced by Fbln1c, human epithelial cells exposed to a Fbln1c peptide had increased CXCL1 mRNA levels compared to medium-exposed controls and a trend towards an increase compared to scrambled peptide-treated controls. Collectively, these results provide strong evidence that Fbln1c plays a key role in airway remodelling, impaired lung function and inflammation in experimental chronic asthma.

We performed a time course study of the development of HDM-induced experimental asthma, and measured the levels of collagen deposition around the small airways and Col1a1, Fbln1 and Fbln1c proteins in whole lungs.
Collagen deposition around the small airways started to occur after 21 days, and Col1a1 proteins were increased after 28 days of HDM challenge. Fbln1 and Fbln1c proteins were also increased after 28 days. However, intranasal treatment with Fbln1c-targeted siRNA from day 21 to day 35 reduced collagen deposition around the airways and in whole lungs after HDM challenge. These data suggest that the role of Fbln1c is to stabilise the formation of collagen in lungs after HDM challenge.

The increased collagen deposition around the small airways of WT mice following 35 days of chronic HDM challenge was not observed in Fbln1c−/− mice. Fbln1c is important in stabilising ECM protein deposition by binding to multiple ECM targets to maintain the structure of the airways and lungs [22]. We show that increases in Fn and Postn in the airways and lung tissue in experimental chronic asthma are dependent on Fbln1c. This suggests that Fbln1c may directly or indirectly bind to multiple ECM targets, and organises collagen deposition in chronic asthma. Fbln1 is known to directly interact with some ECM proteins, such as Fn [44,45]. Fbln1 binds to the heparin II domain in Fn [46], and promotes cell adhesion and has motility-suppressive effects on Fn-coated substrates [47]. Increased levels of Fn proteins have been found around the airways of asthmatic patients [12], and this is reflected in our HDM-induced chronic asthma model. A recent study demonstrated increased levels of Postn protein in the lungs of asthma patients [48]. Furthermore, both Fn and Postn bind to collagen and Fbln1, and Postn and Tnc co-localise in skin keratinocytes [19,49,50]. The reduction of Fn in whole lungs of Fbln1c−/− mice after HDM challenge likely reflects the importance of Fbln1c in stabilising Fn fibres in the ECM matrix. Peribronchial
Fn was reduced after HDM challenge in Fbln1c−/− mice, and the amount of Fn was reduced in the whole lungs even compared to saline-challenged controls. In WT mice we found decreased levels of Tnc protein in whole lungs, but increases around airways after HDM challenge. Our previous study also demonstrated that the levels of ECM protein were different between airways and parenchyma in Fbln1c−/− mice with experimental chronic obstructive pulmonary disease (COPD) [22]. The observation of different changes in the airways compared to whole lungs suggest that the amount of ECM proteins may be produced differently in different regions, such as airways and parenchyma. Indeed, fibroblasts/myofibroblasts, ASM cells, and epithelial cells are major sources of Fn around airways [21,42], however, fibroblasts and alveolar epithelial cells are the primary sources in the parenchyma [51].

We previously showed that fibroblasts [42], bronchoepithelial [22] and ASM cells [21] all produce Fbln1 in the airways and lungs. In this study, we show that Fbln1c regulates the proliferation of ASM cells and fibroblasts/myofibroblasts around the airways and lungs. α-SMA is a marker for both ASM cells and differentiated fibroblasts (myofibroblasts) [52,53], and lower numbers of α-SMA positive cells in Fbln1c−/− mice suggests that the numbers of either or both of these cell types were reduced. In this study, we also show that Fbln1c regulates ASM contractile activity. As well as contraction, ASM cells play important roles in inflammatory responses (e.g., by releasing mast cell mediators [54]). The numbers of ASM cells are also increased in asthma patients, which possibly results from their increased proliferative properties [55,56]. ASM cells also produce and have altered responses to ECM proteins in asthma [13], and the proliferation of these cells
from asthma patients is reduced after targeting of \textit{Fbln1c} mRNA [21]. Fibroblasts/myofibroblasts are major cellular sources of ECM proteins [57], and are increased in the lung tissues of asthmatic children compared to healthy controls [58]. Myofibroblasts in particular are a major producer of type I and III collagen [59]. Thus, a potential decrease in ASM and/or myofibroblasts in our study may be the cause of the reduced collagen deposition in \textit{Fbln1c}\textsuperscript{-/-} mice.

Mucins play critical roles in mucus secretion and AHR in asthma, and previous studies have shown that genetic deletion of \textit{MUC5AC} prevented AAD-induced AHR in mice [60]. We show that \textit{Fbln1c}\textsuperscript{-/-} mice had reduced MUC5AC but not MUC5B proteins in airways compared to WT controls. While increased mucus secretion in the lungs occurs in asthma [61], we found that the numbers of mucus secreting cells were not different in \textit{Fbln1c}\textsuperscript{-/-} compared to WT mice with chronic experimental asthma. The mechanism behind this remains unclear.

Alterations in individual ECM proteins, such as Fbln1c, Tnc, and Postn, change the overall physical properties of the matrix affecting inflammatory cell attachment and migration, as well as the ability of the matrix to bind cytokines that contribute to inflammation. TNF and IL-33 are profibrotic cytokines, involved in airway remodelling [62,63], while the chemokine CXCL1 contributes to the inflammatory process in asthma [64]. WT mice challenged with HDM exhibited significant increases in asthma related cytokines (IL-5, IL-13, IL-33, and TNF) in their lungs and lung draining lymph nodes. However, depletion of \textit{Fbln1c} in mice protected against increases in these factors in chronic experimental asthma. We demonstrate that there were fewer GATA3\textsuperscript{+}
T cells in the lymph nodes and lungs of mice treated with Fbln1c siRNA compared to scrambled siRNA. We also showed treatment reduced DC-induced Th2 responses. This indicates that Fbln1c modifies DC and T cell function to promote DC-induced Th2 responses. However, the mechanism remains unclear. The decreased inflammation in mice after inhibition of Fbln1c may be the result of direct effects on the cellular expression of these cytokines and chemokines, or may occur indirectly through reductions in other immunomodulatory factors, such as Tnc and Postn. Recent studies demonstrate that Tnc−/− and Postn−/− mice have reduced lung inflammation in mouse models of asthma [65,66]. Previous studies showed that Fbln1 is associated with heparin-binding epidermal growth factor (EGF)-like growth factor, indicating that it may induce the EGF receptor signalling pathway to promote inflammation [67]. Fbln1 may also bind to integrin β1 suggesting a role for the integrin signalling pathway [67]. However, Fbln1c has not yet formally been shown to bind to these proteins. To further assess how Fbln1c might be controlling inflammation, we attempted an immunoprecipitation to identify its binding partners on inflammatory cell surfaces (data not shown). However, this failed due to the lack of a suitable Fbln1c antibody.

CXCL1 is an important chemokine that is released from epithelial cells [68], and is involved in regulating inflammatory cells and ASM migration in asthma [69,70]. Fbln1c−/− mice had significantly lower levels of CXCL1 protein in lung tissue compared to WT mice. Although the exact mechanism remains unclear, we show that Fbln1c peptide increased CXCL1 mRNA levels in two human epithelial cell lines compared to vehicle but only showed non-statistically significant trends to increases compared to scrambled peptide-
treated controls. We interpret these data to indicate that Fbln1c causes epithelial cells to secrete chemokines to induce inflammatory responses.

Collectively, our data suggest that Fbln1c plays a key role in regulating airway remodelling and inflammation in chronic asthma. Fbln1c stabilises ECM protein deposition, including Fn and Postn, and regulates collagen levels around the airways and in lungs that results in airway remodelling and AHR in HDM-induced chronic experimental asthma. Fbln1c is required for increases in α-SMA positive cells around airways that regulate ASM contraction. Fbln1c also regulates airway inflammation associated with the influx of neutrophils, eosinophils and lymphocytes, and their related cytokines and chemokines with HDM challenge. Fbln1c plays a central role in the process of ECM deposition and inflammatory responses, and may be a novel therapeutic target for the inhibition of airway remodelling and inflammation in chronic asthma.

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Author contributions statement

G.L, A.G.J, J.K.B and P.M.H participated in design of the study. G.L performed all *in vivo* and most of *in vitro* experiments. M.A.C and W.S.A generated *Fbln1c*−/− mice. P.M.N and T.J.H assisted with mouse experiments. C.D, J.E.B and C.L.G helped perform mouse airway contractility studies. A.C.H and P.A.W assisted in experiments using human minimally immortalised epithelium-derived basal (BCi-NS1.1) cells. A.C.B, H.T and P.S.F assisted in T cell isolation and flow cytometry. G.L, M.A.C, Q.G, N.G.H, J.C.H, B.G.O, D.A.K, J.K.B and P.M.H contributed to preparing and editing the manuscript and for intellectual input. All authors read and approved the final manuscript (with the exception of W.S.A, who passed away before the final version was completed).
References


Figure legends

Figure 1. Chronic house dust mite (HDM) challenge results in airway remodelling and airway hyperresponsiveness (AHR) in association with increased Fbln1 and Fbln1c protein levels in the lungs in experimental chronic asthma. (A) Mice were chronically exposed to HDM via the airways for 35 days. Controls received saline. (B) A time course of lung sections was stained with Sirius Red and Fast Green (left, scale bar = 50 μm), and collagen area around the small airways was quantified and normalised to the perimeter of the basement membrane (Pbm). (C) Time course of type I collagen (Col1a1) in whole lungs by immunoblot. (D) Airway resistance (left) and dynamic compliance (right) were measured in response to increasing concentrations of methacholine after 35 days of HDM challenge. Time course of Fbln1 (E) and Fbln1c (F) protein levels assessed in whole lung homogenates by immunoblot (top), and fold change of densitometry normalised to β-actin (bottom). Results are mean ± SEM. n=6-8 mice per group. *P<0.05, **P<0.01, ***P<0.001 compared to saline-challenged WT controls.

Figure 2. Genetic deletion and therapeutic targeting of Fbln1c in mice protects against increased collagen deposition around the small airways and in whole lungs, and airway hyperresponsiveness (AHR) in house dust mite (HDM)-induced experimental chronic asthma. WT and Fbln1c−/− mice were administered HDM to the airways for 35 days. Controls received saline. (A) Lung sections were stained with Sirius Red and Fast Green (left, scale bar = 500 μm; insets show expanded images of indicated regions; scale bar = 50 μm). Collagen area around small airways was normalised to the perimeter of
basement membrane (Pbm, right). (B) Total collagen levels were assessed by measuring hydroxyproline (left), and soluble collagen (right) in whole lungs. (C) Type I collagen (Col1a1) protein was measured in whole lungs by immunoblot (left), and fold-change of type I collagen was normalised to β-actin and compared to saline-challenged WT controls (right). (D) AHR in terms of airway resistance in response to increasing concentrations of methacholine challenge (left), and a representative plot of airway resistance at the maximal dose (50 mg/ml) of methacholine (right). (E) AHR in terms of dynamic compliance in response to increasing concentrations of methacholine challenge (left), and a representative plot of dynamic compliance at 50 mg/ml of methacholine (right). Mice were administered HDM to the airways for 35 days to induce experimental asthma and were treated with Fbln1c-targeted or scrambled siRNA from day 21 to day 35. (F) Collagen was stained with Sirius Red and Fast Green (left, scale bar = 50 μm) and normalised to Pbm (right). (G) Col1a1 proteins in whole lungs (left), and fold change of Col1a1 were normalised to β-actin (right). Airway resistance (H) and dynamic compliance (I) in response to increasing concentrations of methacholine challenge. Results are mean ± SEM. n=6–8 mice per group. *P<0.05, **P<0.01, ****P<0.0001 compared to saline-challenged WT or Fbln1c−/− controls. #P<0.05, ##P<0.01, ####P<0.0001 compared to HDM-challenged WT controls. $P<0.05, $$P<0.01 compared to HDM-challenged controls with scrambled siRNA.

Figure 3. Fbln1c−/− mice are partially or completely protected against increased fibronectin (Fn) and periostin (Postn) but increased tenascin-c (Tnc)
deposition is unaffected around the small airways or in whole lungs in house dust mite (HDM)-induced experimental chronic asthma. (A) Fn, (B) Tnc, and (C) Postn deposition around small airways was assessed by immunohistochemistry (left) scale bars, 50 μm, and quantification of the areas was normalised to the perimeter of the basement membrane (Pbm) (right). (D) Protein levels in whole lungs was assessed by immunoblot (left), and fold change determined using densitometry normalised to β-actin and compared to saline-challenged WT controls (right). Results are mean ± SEM. n=6–8 mice per group. *P<0.05, **P<0.01 ***P<0.001, ****P<0.0001 compared to saline-challenged WT or Fbln1c−/− controls. #P<0.05, ##P<0.01, ###P<0.001, ####P<0.0001 compared to HDM-challenged WT controls. NS is non-significant.

**Figure 4.** Fbln1c−/− mice are protected against increases in α-smooth muscle actin (α-SMA) positive cells around medium and small airways, and exaggerated airway contractility in house dust mite (HDM)-induced experimental chronic asthma. α-SMA (red) and nuclei (blue) staining around (A) medium and (B) small airways (left) scale bars = 200 μm, insets show expanded image of indicated regions; scale bars = 50 μm. Quantification of α-SMA area was normalized to the perimeter of the basement membrane (Pbm, right). (C) Frame-by-frame analysis showing changes in airway lumen area with methacholine (MCh) exposure. (D) Average contraction over the last minute of perfusion of each concentration of MCh exposure. Results are mean ± SEM. n=6–8 airways from n=5–8 mice per group. *P<0.05 compared
to saline-challenged WT or Fbln1c−/− controls. #P<0.05 compared to HDM-challenged WT controls.

**Figure 5.** Genetic and therapeutical inhibition of Fbln1c in mice are protected against pulmonary inflammation in house dust mite (HDM)-induced experimental chronic asthma. WT and Fbln1c−/− mice were administered chronic HDM for 35 days. Controls received saline. (A) Differential inflammatory cell counts in bronchoalveolar lavage fluid (BALF). (B) IL-5, (C) IL-13, (D) IL-33, (E) TNF and (F) CXCL1 protein levels in whole lung homogenates measured by ELISA. Mice were administered HDM for 35 days to induce experimental asthma and were treated with Fbln1c-targeted or scrambled siRNA or vehicle. (G) Differential inflammatory cell counts in BALF. (H) IL-5, (I) IL-13, (J) IL-33, (K) TNF and (L) CXCL1 protein levels in whole lung homogenates. GATA3+ Th2 cells from lymph nodes (M) and lungs (N) were enumerated by flow cytometry. Results are mean ± SEM. n=6–8 mice per group. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 compared to saline- or dH2O-treated WT or Fbln1c−/− controls. #P<0.05, ##P<0.01, ###P<0.0001 compared to HDM-treated WT controls. $P<0.05, $$P<0.01 compared to HDM controls treated with scrambled siRNA.

**Figure 6** Genetic and therapeutical depletion of Fbln1c in mice protects against increased Th2 cytokine and chemokine production in lung-draining lymph nodes in house dust mite (HDM)-induced experimental chronic asthma, as well as Fbln1c peptide induced CXCL1 in a human epithelial cell line. Cells were isolated from the lymph nodes of WT and Fbln1c−/− mice, cultured with
HDM and (A) IL-5, (B) IL-13, and (C) TNF protein levels in supernatants measured by ELISA. Mice were sensitised and challenged with HDM for 35 days to induce experimental asthma and were treated with *Fbln1c*-targeted or scrambled siRNA. Dendritic cells and T cells were isolated from lymph nodes, re-stimulated with HDM, and IL-5 (D) and IL-13 protein levels in supernatants were measured by ELISA. n=6–8 mice per group. Human epithelial (A549) cells (F) and human epithelium-derived basal (BCi-NS1.1) cells (G) were cultured with Fbln1c peptide or scrambled peptide or were uncoated and contained media only and *CXCL1* mRNA levels were measured by Reverse Transcription-qPCR. n=6. Results are mean ± SEM. *P<0.05, ****P<0.0001 compared to saline-challenged WT or *Fbln1c*−/− controls with HDM re-stimulation. #P<0.05, ##P<0.01 compared to HDM-challenged WT mice with HDM re-stimulation. &P<0.05, &&P<0.01 compared to HDM-challenged mice with scrambled siRNA. ØP<0.05, ØØP<0.01, compared to culture medium control.
Figure S1. Fbln1c deficiency does not change the numbers of mucus secreting cells around the airways in HDM-induced experimental chronic asthma

Figure S2. The absence of Fbln1c reduced MUC5AC but not MUC5B area around the airways in HDM-induced experimental chronic asthma

Figure S3. GATA3+ Th2 cell numbers were not different between naive WT and Fbln1c−/− mice

Figure S4. Targeting of Fbln1c with siRNA reduced CD45+ CD4+ CD3+ GATA3+ Th2 cell number in lymph nodes in HDM-induced experimental chronic asthma

Figure S5. Targeting of Fbln1c with siRNA reduced CD45+ CD4+ CD3+ GATA3+ Th2 cells in the lungs in HDM-induced experimental chronic asthma
Supplementary Method and Materials

Reference numbers refer to the main text list

Airway remodelling

Isolated mouse lungs were perfused with 0.9% saline at fixed pressure, formalin-fixed, paraffin-embedded, sectioned and stained with Sirus Red and Fast Green. Photomicrographs were taken using an Aperio AT2 image scanner (Leica Biosystems, Wetzlar, Hesse, Germany). Images were evaluated and airway remodelling was analysed using ImageJ (version 1.47) as described previously [22,24]. Airways were divided into three categories according to the perimeter of their basement membrane (Pbm): Pbm ≤1 mm (small), Pbm >1 to ≤2 mm (medium) and Pbm > 2mm (large). At least 6 airways per mouse were blind-selected from 4-6 mice in each experimental group. The widths of the perimeter of the basement membrane (Pbm), the inner collagen area (Ai), and the outer collagen area (Ao) were measured using ImageJ. Collagen area (Wct) was calculated (Wct = Ao-Ai) and normalised to the Pbm.

AHR

Mice were anesthetized with ketamine (40mg/kg, Ceva Animal Health Pty Ltd, Glenorie, NSW, Australia) and xylazine (10mg/kg, Troy Laboratories, Smithfield, NSW, Australia). Mice were cannulated and attached to a ventilator (Buxco Electronics, Sharon, CT, USA). Airway resistance (R_L) and dynamic compliance (C_Dyn) were assessed by analysis of pressure and flow waveforms in response to increasing doses of methacholine.
Protein extraction

Lung tissues were homogenised in radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich, Castle Hill, NSW, Australia) or PBS supplemented with PhosSTOP phosphatase inhibitor and complete protease inhibitor cocktails (Roche, Mannheim, Germany) as described previously [28,29,31]. Tissue debris was removed by centrifugation (8,000 x g, 10 min, 4 °C), and proteins were collected for analysis using immunoblot or ELISA. Protein concentrations were determined using a BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA).

Immunoblotting

Proteins were separated by SDS-PAGE electrophoresis using Mini-PROTEAN TGX Stain-Free gels (Bio-Rad, USA), and transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). Proteins were detected using antibodies, including Fbln1c obtained as described previously [17], Fbln1 (ab175204, Abcam, Milton, CB, UK), Col1a1 (ab21286, Abcam), Fn (F3648, Sigma-Aldrich, St. Louis, MO, USA), Postn (ab14041, Abcam), Tnc (sc-20932, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and β-actin (ab70165, Abcam). Images of immunoblots were captured with a ChemiDoc MP System (Bio-Rad Laboratories, Hercules, CA, USA). Blots were cut based on protein molecular weights, so that multiple proteins could be detected at the same time. The densitometric values of proteins of interest were measured and normalised according to the density of internal control proteins, such as β-actin, using ImageJ as described
previously [37]. Values are represented as fold-change of the experimental
groups compared to control groups.

**Hydroxyproline assay**

Lung tissues were homogenised in 6N HCl at 130 °C for 8 h. Samples were
incubated with chloramine-T solution (1.4% chloramine-T, 10% N-propanol
and 80% citrate-acetate buffer [110 mM sodium acetate trihydrate, 20 mM
citric acid, 75 mM sodium hydroxide, pH 6.5]) at room temperature for 20 min,
and incubated with Ehrlich’s solution at 65 °C for 18 min. Absorbance was
measured at 558 nm. The concentrations of hydroxyproline (Sigma-Aldrich)
were determined against standard curves generated using known
concentrations of pure hydroxyproline.

**Soluble collagen assay**

Soluble collagen was determined using Sircol Collagen Assay kits (Biocolor,
Carrickfergus, BT, UK) according to the manufacturer’s instructions as
described previously [22].

**Mucus secreting cells**

Sections of mouse lung sections were deparaffinised and stained with
Periodic acid-Schiff (PAS) using a commercially available kit (395B-1KT,
Sigma-Aldrich) according to the manufacturer’s instructions; sections were
immersed in periodic acid solution for 5 min at room temperature and
following three rinses with distilled water incubated with Schiff’s reagent for 15
min at room temperature, then counterstained with haematoxylin for 90 s. Mucus and goblet cells stain purple/magenta, whereas nuclear material stains blue. The number of PAS-positive cells per 100 μm length of airways was enumerated under high-power magnification (100x) as described previously [34].

Immunostaining
Longitudinal sections of mouse lungs were incubated with citrate buffer (10mM, pH6, 100 °C, 35 min) for antigen retrieval. Non-specific binding was blocked with casein (5%, B6429, Sigma-Aldrich, USA) at room temperature for 1 h. Slides were incubated with antibodies raised against Fbn1 (1:4,000, Abcam), Fn (1:2,000, Sigma-Aldrich, USA), Postn (1:2,000, Abcam) or Tnc (1:4,000, Santa Cruz Biotechnology) at 4 °C overnight, followed by anti-rabbit horseradish peroxidase-conjugated secondary antibody (HAF008, R&D Systems, Minneapolis, MN, USA) at 37 °C for 30 min. Diaminobenzidine (DAB, DAKO, Australia) was used as the chromogen and haematoxylin as a nuclear counterstain.

Immunofluorescence
Longitudinal sections of mouse lungs were deparaffinised and incubated with citrate buffer (10mM, pH6, 100 °C, 35 min) for antigen retrieval. Non-specific binding was blocked with casein (5%, B6429, Sigma-Aldrich, USA) at room temperature for 1 h. Slides were incubated with anti-MUC5AC (1:100, ab3649, Abcam), or anti-MUC5B (1:200, sc-21768, Santa Cruz Biotechnology) antibodies at 4 °C overnight, followed by Cy3-conjugated anti-
rabbit secondary antibody (R&D Systems) as described previously [60]. Some slides were incubated with an α-SMA antibody conjugated with Cy3 (c6198, Sigma-Aldrich) at 4°C overnight. Hoechst 33258 (1:200, 94403, Sigma-Aldrich) was used to stain nuclei in the sections. The area of proteins was normalised to the length of basement membrane as described previously [22,60].

**Airway contractility**

Isolated mouse lungs were inflated with 2% agarose gel dissolved in Hank's balanced salt solution, sectioned at 150 μm using a vibratome (VT 1000S, Leica), and incubated overnight in DMEM supplemented with 1% penicillin-streptomycin. Lung slices were mounted in a custom-built perfusion chamber and perfused with increasing concentrations of methacholine. Dilator responses were assessed in airways pre-contracted with methacholine as described previously [35,36].

**Bronchoalveolar lavage fluid (BALF)**

BALF was collected as described previously [22]. In brief, the multi-lobed lung was tied off and BALF was collected from the single-lobed lung by washing twice with PBS (500 μl). Cells were pelleted (150xg, 10 min) and resuspended in red blood cell lysis buffer. Remaining cells were cytocentrifuged (300 x g, 5 min, ThermoFisher Scientific, Australia) onto microscope slides. Cells were stained with May-Grünwald-Giemsa and enumerated according to morphological criteria using a light microscope as described previously [38].
ELISA
Concentrations of IL-5, IL-13, IL-33, TNF and CXCL1 proteins in lung tissue homogenates were determined using duoset ELISA kits (R&D Systems, Gymea, Australia) according to the manufacturer's instructions and as described previously [27,37].

Lymph node and lung assays
Cells from mouse lungs and draining lymph nodes were collected and filtered as described previously [39]. The cells were cultured with or without 5 μg/ml HDM for five days, and the levels of secreted IL-5 and IL-13 were measured by ELISA [27,38]. Some cells were stained with Th2 cell markers and enumerated by flow cytometry.

Flow cytometry
Single cell suspensions were prepared from mouse lungs and lymph nodes as described previously [31]. Cells were incubated with Fc-blocking buffer in the dark at room temperature for 30 min to prevent non-specific binding, then stained with CD4 conjugated to PE (60029, Stemcell Technologies, Vancouver, BC, Canada), CD3 conjugated to APC (100312., Biolegend, San Diego, CA, USA), and CD45 conjugated to PerCpCy5.5 (109828., Biolegend) antibodies according to the manufacturer’s instructions. Cells were then permeabilised and stained intracellularly with anti-GATA3 conjugated to PEcy7 (560405, BD Biosciences, Franklin Lakes, NJ, USA). Flow cytometric analysis was performed using a BD LSRFortessa X-20 Cytometer with
FACSDiva software (BD Biosciences). Data was analysed using FlowJo software (Tree Star, Inc., Ashland, Oregon, USA) as described [40].

Dendritic cell (DC) and T cell cytokine release

Mice in chronic HDM-induced experimental asthma and control groups were treated with Fbln1c-targeted siRNA or scrambled siRNA or vehicle. DCs and T cells were isolated from lung draining lymph nodes using EasySep kits (Stemcell Technologies, USA) according to the manufacturer's instructions. DCs and T cells were co-cultured in DMEM medium with or without 5 μg/ml HDM for 5 days, and the levels of secreted IL-5 and IL-13 were measured by ELISA [39, 40].

In vitro experiments

Cells of the human airway epithelial A549 line and epithelium derived from basal (BCi-NS1.1) cells [41] were cultured in Dulbecco's modified Eagle's medium (DMEM, D5671, Sigma-Aldrich, USA) containing 5% fetal bovine serum (FBS, Bovogen, East Keilor, VIC, Australia), 25 mM hydroxyethylpiperazine-N'-2-ethanesulphonic acid buffer, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a 5% CO₂ incubator. Fbln1c peptide (RCERLPCHENRECSKLPLRI) was obtained as described previously [43], and scrambled peptide (CCLECRRHRESNKPIRLLEP) was obtained from GL Biochem (Shanghai, China). Six-well plates were coated with human Fbln1c peptide (100 μg/ml) at room temperature, overnight. After three PBS washes, A549 or BCi-NS1.1 cells were seeded into wells with DMEM containing 0.1% FBS. Cells were lysed and RNA was extracted after 24 and 48 h. Total RNA
was reversed transcribed as described previously [41] and mRNA abundance determined by real-time quantitative PCR (qPCR) using human CXCL1 primers (F:GCCAGTGCTTGACAGACCT; R:GGCTATGACTTCGGTTTGGG), and expressed relative to the level of GAPDH mRNA (F: GTCGCTGTGGAGTCAGAGG; R: GAAACTGTGGCGTATGG).

Statistics

For mouse studies, results are presented as mean ± standard error of the mean (SEM) from 4–8 mice, in duplicate or triplicate experiments. Cell culture experiments were repeated at least three times for each time point. The statistical significance of differences between two group means was determined using two-tailed Student’s t-tests. Comparisons involving more than two groups used one-way or two-way analysis of variance (ANOVA) and Bonferroni post-test correction. GraphPad-Prism Software (version 6, GraphPad, La Jolla, CA, USA) was used to plot the data.