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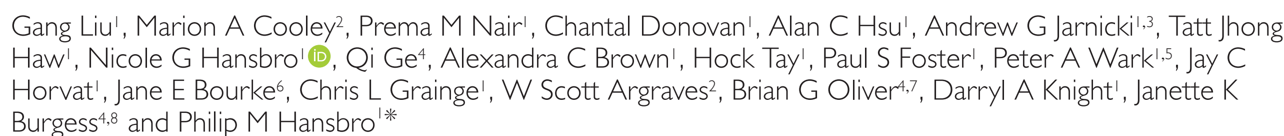
ORIGINAL PAPER

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 characterized by allergic airway inflammation, airway remodelling, and airway 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 stabilizes collagen and other ECM proteins. The level of 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 the level of Fbln1c was increased in the lungs of mice with house dust mite (HDM)-induced chronic allergic airway disease (AAD). Genetic deletion of *Fbln1c* and therapeutic inhibition of Fbln1c in mice with chronic AAD reduced airway collagen deposition, and protected against AHR. *Fbln1c*-deficient (*Fbln1c*$^{-/-}$) mice had reduced mucin (MUC) 5 AC levels, but not MUC5B levels, in the airways as compared with 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 numbers of α-smooth muscle actin-positive cells around the airways and reduced airway contractility as compared with WT mice. After HDM challenge, these mice also had fewer airway inflammatory cells, reduced interleukin (IL)-5, IL-13, IL-33, tumour necrosis factor (TNF) and CXCL1 levels in the lungs, and reduced IL-5, IL-33 and TNF levels in lung-draining lymph nodes. Therapeutic targeting of Fbln1c reduced the numbers of GATA3-positive Th2 cells in the lymph nodes and lungs after chronic HDM challenge. Treatment also reduced the secretion of IL-5 and IL-13 from co-cultured dendritic cells and T cells restimulated with HDM extract. Human epithelial cells cultured with Fbln1c peptide produced more *CXCL1* mRNA than medium-treated controls. Our data show that Fbln1c may be a therapeutic target in chronic asthma.
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Keywords: asthma; allergic airway disease; fibulin-1; collagen; fibrosis; airway remodelling; inflammation; lung function; airway hyperresponsiveness

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No conflicts of interest were declared.

Introduction

Asthma is a chronic inflammatory respiratory disease predominantly of the airways. It is characterized by airway inflammation and remodelling that leads to airway hyperresponsiveness (AHR) and reversible airflow obstruction [1,2]. Clinical symptoms include shortness of breath, wheezing, chest tightness, and dry cough [3]. There are >300 million people suffering from asthma worldwide [4]. Asthma exacerbations constitute a major problem, causing increases in symptoms and hospitalizations, and are triggered by multiple allergic and exogenous stimuli, such as respiratory infections, house dust mites (HDMs), pollen, and occupational chemicals

[5,6]. The symptoms of disease can be controlled in people with mild to moderate allergic asthma by the use of combination therapies with inhaled corticosteroids and long-acting β-agonists; new biologics such as omalizumab (anti-IgE antibody), dupilumab [anti-interleukin (IL)-4Rα antibody] and mepolizumab (anti-IL-5) are also showing promise [1,2]. However, these medications only treat the symptoms; the underlying disease features, in particular chronic airway remodelling, are not altered.

Airway remodelling is an important feature of chronic asthma; it 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 specific asthma endotypes [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 be present 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 stabilization of other ECM proteins, including Fn, Postn, and Tnc, in the lung [18,19]. There are four variants of Fbln1 (Fbln1a, Fbln1b, Fbln1c, and Fbln1d) in humans, with each one having a different C-terminal sequence. Fbln1c and Fbln1d are the predominant variants in adult humans and mice [20]. We have shown that the levels of total Fbln1 are increased in the serum and bronchoalveolar lavage fluid (BALF) of asthma patients as compared with non-asthmatic subjects [21]. Our *in vitro* studies have shown that stimulation of ASM cells from asthmatic patients with transforming growth factor-β increases the levels of secreted Fbln1 [21]. However, the levels specifically of Fbln1c in asthma patients are unknown. We have also shown that antisense oligonucleotide silencing of *Flbn1c* reduces ASM cell proliferation [21], and that *Fbln1c*-deficient (*Fbln1c*$^{-/-}$) 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

Female wild-type (WT) or *Fbln1c*$^{-/-}$ C57BL/6 J mice aged 6–8 weeks 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 of sterile saline for five consecutive days per week for 5 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 of *Fbln1c* small interfering RNA (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 deparaffinized, and collagen was stained with 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 with ImageJ (version 1.47; Media Cybernetics, Rockville, MD, USA) [22,24]. AHR was measured as described previously [25–27].

Protein extraction, and immunoblotting, hydroxyproline and soluble collagen assays

Lung tissues were homogenized, proteins were detected with antibodies, and 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 numbers of mucus-secreting cells around the airways were counted [29,34].

Immunostaining and immunofluorescence assays

Mouse lung sections were incubated with antibodies as described previously [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

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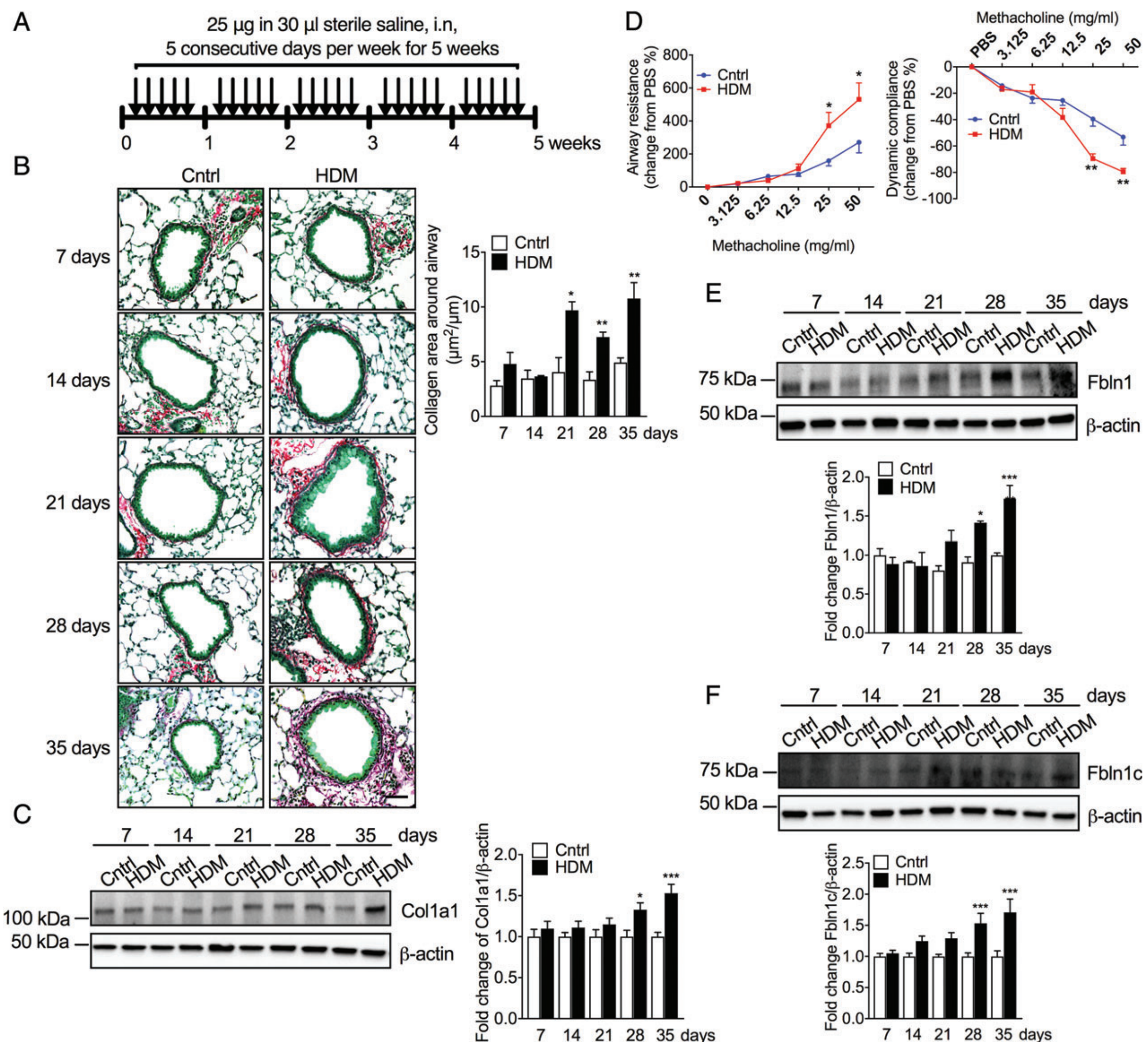

Figure 1. Chronic HDM challenge results in airway remodelling and AHR in association with increased Fbln1 and Fbln1c levels in the lungs in experimental chronic asthma. (A) Mice were chronically exposed to HDM extract via the airways for 35 days. Controls received saline. (B) A time course of lung sections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

airway contractility was assessed as described previously [35,36].

BALF assays and enzyme-linked immunosorbent assays (ELISAs)

BALF was collected, and differential leukocyte counts were determined. Cytokines and chemokines were assessed from mouse lungs by ELISA 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 extract for 5 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 and 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 cells and minimally immortalized epithelium-derived basal (BCi-NS1.1) cells were cultured with Fbln1c peptide and scrambled peptide as described previously [41]; RNA extraction

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and reverse transcription quantitative polymerase chain reaction (qPCR) were performed as described previously [42,43] and in supplementary material, Supplementary materials and methods.

Statistics

Data are presented as mean ± standard error of the mean (SEM) from four to eight 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 ASM cells 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 extract from 21 days to 35 days as compared with 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 of HDM challenge (Figure 1C). These mice also developed AHR, characterized by increased airway resistance and decreased dynamic compliance in response to increasing concentrations of methacholine, as compared with controls, after 35 days of HDM challenge (Figure 1D). Fbln1 and Fbln1c levels were significantly increased in the lung tissue from 28 to 35 days of HDM challenge as compared with controls, as assessed by immunoblotting (Figure 1E, 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*$^{-/-}$ mice were created as previously described [22], and chronically exposed to HDM extract. The absence of *Fbln1c* resulted in complete abolition of the deposition of collagen around the small airways as compared with WT mice after chronic HDM challenge (Figure 2A), and our previous study showed similar data [22]. Increases in the levels of total and soluble collagen in lung tissue (Figure 2B), and levels of Col1a1, were completely abolished (Figure 2C) in *Fbln1c*$^{-/-}$ mice. Indeed, the levels of collagen deposition, total and soluble collagen and Col1a1 in *Fbln1c*$^{-/-}$ mice exposed to HDM extract 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 are also chronic features of asthma. Thus, we assessed the impact of the absence of Fbln1c on these features. 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 as compared with their controls. However, there were no differences between the numbers of PAS-positive cells in the airways of HDM-challenged WT or *Fbln1c*$^{-/-}$ mice (supplementary material, Figure S1A, B). We also assessed the levels of mucin (MUC) 5 AC and MUC5B in the airways by using immunofluorescence. *Fbln1c*$^{-/-}$ mice had significantly reduced MUC5AC but not MUC5B 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 *Fbln1c*$^{-/-}$ 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 *Fbln1c*$^{-/-}$ mice.

We then assessed whether siRNA to suppress the levels of Fbln1c could be used therapeutically. As airway remodelling occurred after 21 days of HDM challenge (Figure 1B), *Fbln1c*-targeted or scrambled siRNA or vehicle were administered every 2 days from day 21 to day 35 of HDM challenge. *Fbln1c* siRNA treatment after the establishment of remodelling reduced the levels of collagen deposition around the small airways (Figure 2F) and Col1a1 (Figure 2G) in the lungs. Immunoblotting was used to distinguish 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 stabilization [22]. Thus, the role of Fbln1c in the deposition of ECM-associated proteins

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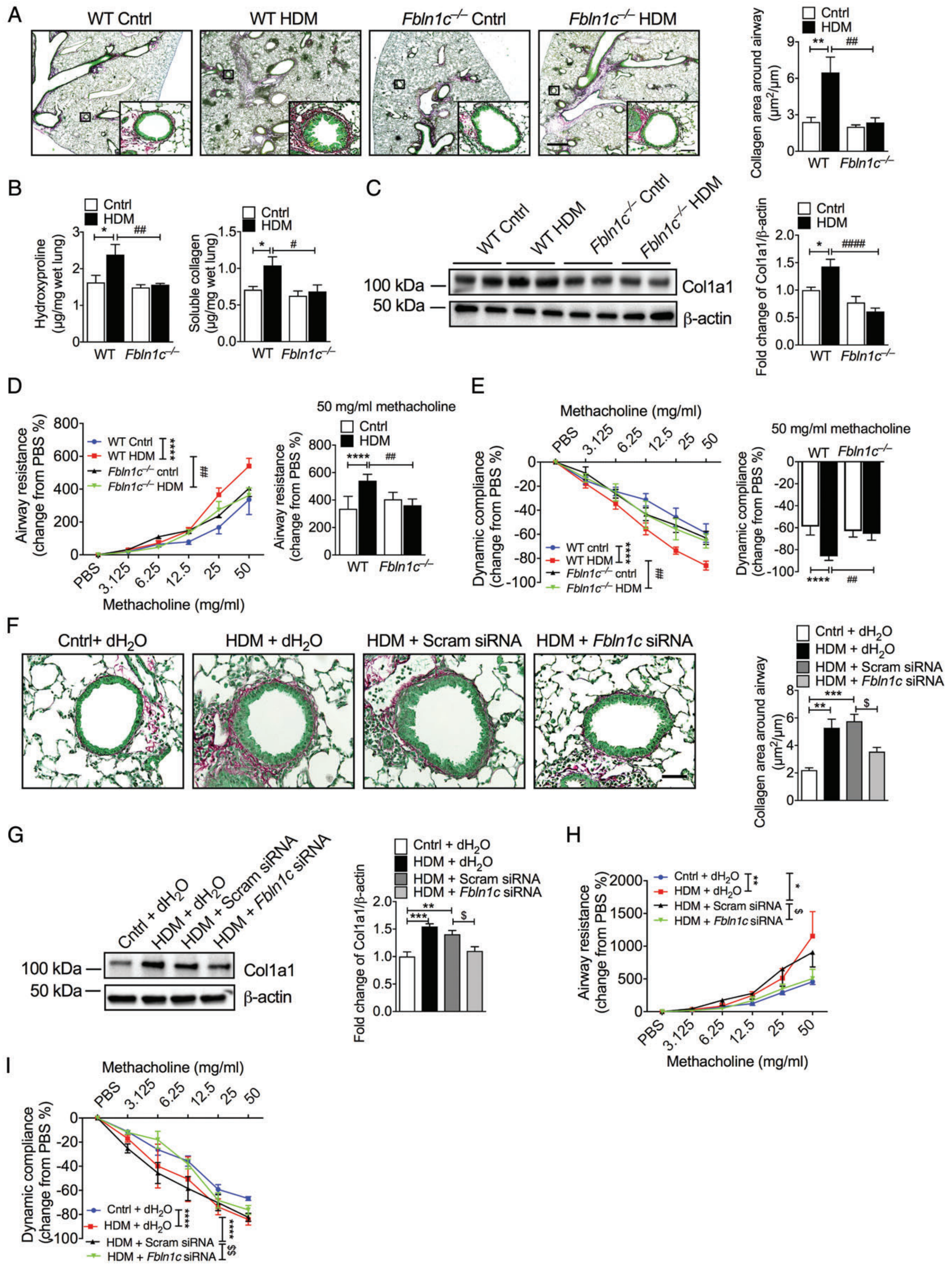

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in HDM-induced chronic asthma was examined. HDM challenge of WT mice resulted in substantial (two-fold) increases in the levels of Fn, Postn and Tnc around the small airways as compared with saline-challenged controls (Figure 3A–C). However, HDM-challenged *Fbln1c*$^{-/-}$ mice showed only partial increases in Fn levels and were completely protected against increases in Postn levels. Tnc levels increased to the same extent as in HDM-exposed *Fbln1c*$^{-/-}$ and WT mice.

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

These data demonstrate that Fbln1c is necessary for the increased levels of Fn and Postn but not of 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

An increased number 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 by using immunofluorescence. Following chronic HDM challenge of WT mice, the numbers of α-SMA-positive cells around the medium (Figure 4A) and small (Figure 4B) airways were doubled as compared with saline-challenged controls. HDM-challenged *Fbln1c*$^{-/-}$ mice were completely protected against these increases in α-SMA-positive cell numbers. Indeed, the numbers of α-SMA-positive cells in *Fbln1c*$^{-/-}$ mice challenged with HDM extract were the same as the baseline numbers in saline-challenged control WT and *Fbln1c*$^{-/-}$ mice.

Increased ASM cell mass results in abnormal airway contractility in asthma. Therefore, we assessed the role of Fbln1c in airway contraction by measuring changes in 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 than in 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 whether Fbln1c had any role in inflammation in chronic experimental asthma. Both total leukocyte numbers and numbers of macrophages, neutrophils, eosinophils and lymphocytes were significantly increased in the BALF of WT mice after chronic HDM challenge (Figure 5A). The numbers of all of these cell types were also increased in HDM-challenged *Fbln1c*$^{-/-}$ mice; however, the numbers of neutrophils, eosinophils and lymphocytes were significantly lower 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 and tumour necrosis factor (TNF), and the chemokine CXCL1, in lung tissue as compared with saline-challenged controls (Figure 5B–F). However,

Figure 2. Genetic deletion of *Fbln1c* and therapeutic targeting of Fbln1c in mice protects against increased collagen deposition around the small airways and in whole lungs, and AHR in HDM-induced experimental chronic asthma. WT and *Fbln1c*$^{-/-}$ mice had HDM extract administered 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. The collagen area around small airways was normalized to the perimeter of basement membrane (Pbm, right). (B) Total collagen levels were assessed by measuring hydroxyproline (left) and soluble collagen (right) levels in whole lungs. (C) The level of Col1a1 was measured in whole lungs by immunoblotting (left), and the fold change of type I collagen was normalized to β-actin and compared with saline-challenged WT controls (right). (D) AHR in terms of airway resistance in response to increasing concentrations of methacholine (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 (left), and a representative plot of dynamic compliance at 50 mg/ml methacholine (right). Mice had HDM extract administered 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), and normalized to Pbm (right). Scale bar: 50 μm. (G) Col1a1 levels in whole lungs (left) and fold change of Col1a1 were normalized to β-actin (right). (H and I) Airway resistance (H) and dynamic compliance (I) in response to increasing concentrations of methacholine. Results are mean ± SEM. $n = 6$–8 mice per group. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{****}P < 0.0001$ as compared with saline-challenged WT or *Fbln1c*$^{-/-}$ controls. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#\#}P < 0.0001$ as compared with HDM-challenged WT controls. $^{\$}P < 0.05$ and $^{\$\$}P < 0.01$ as compared with HDM-challenged controls with scrambled siRNA. Cntrl, control; PBS, phosphate-buffered saline.

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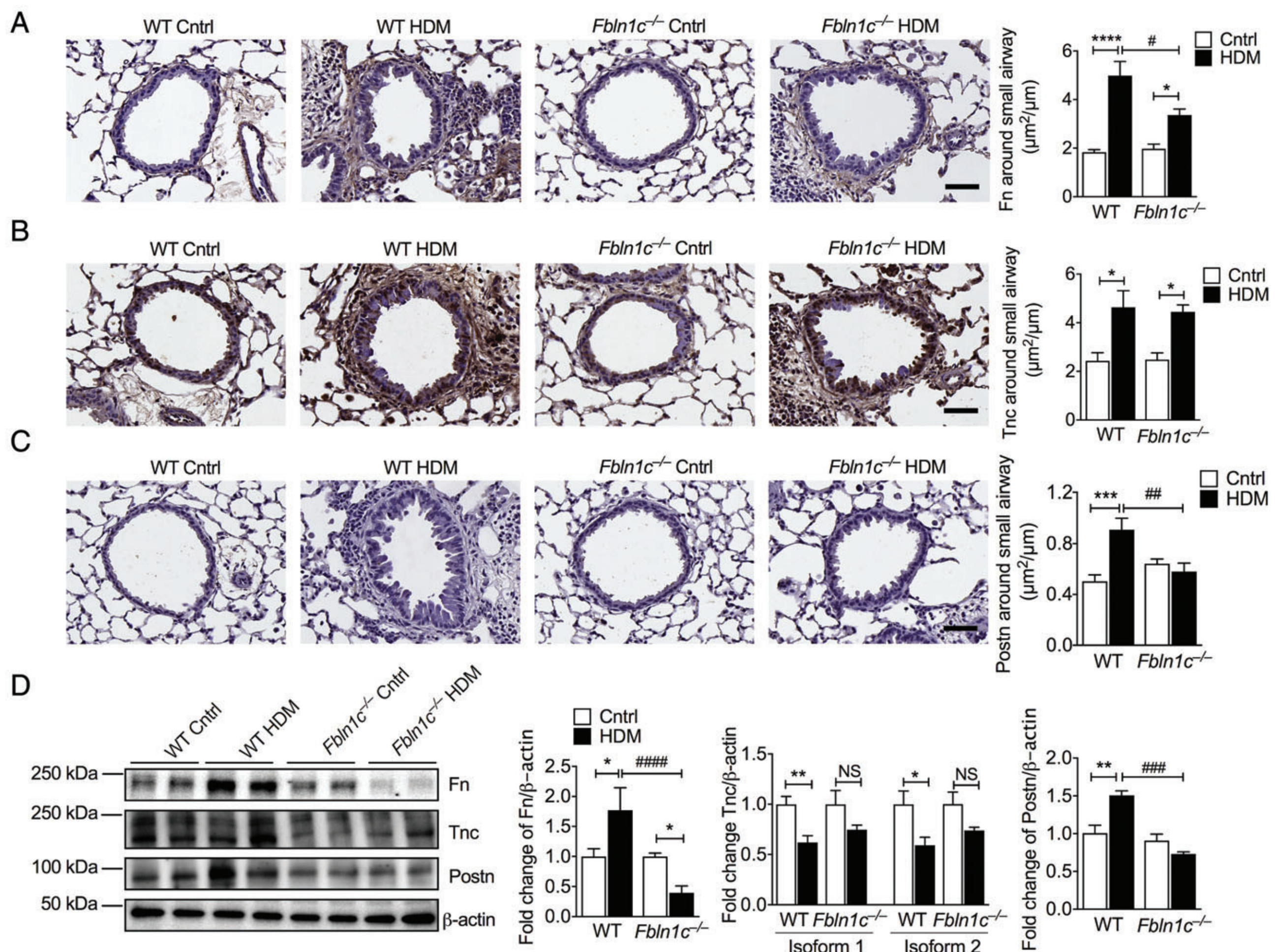

Figure 3. $Fbln1c^{-/-}$ mice are partially or completely protected against increased Fn and Postn deposition, but increased Tnc deposition is unaffected around the small airways or in whole lungs in HDM-induced experimental chronic asthma. (A–C) Fn (A), Tnc (B) and Postn (C) deposition around small airways was assessed by immunohistochemistry (left), and quantification of the areas was normalized to the perimeter of the basement membrane (Pbm) (right). Scale bars: 50 μm. (D) Protein levels in whole lungs were assessed by immunoblotting (left), and fold change was determined by the use of densitometry normalized to β-actin and compared with saline-challenged WT controls (right). Results are mean ± SEM. $n = 6–8$ mice per group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$ as compared with saline-challenged WT or $Fbln1c^{-/-}$ controls. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ and $^{\#\#\#\#}P < 0.0001$ as compared with HDM-challenged WT controls. Cntrl, control; NS, non-significant.

$Fbln1c^{-/-}$ mice were completely protected against HDM-induced increases in these factors, with levels being no different to those in saline-challenged WT and $Fbln1c^{-/-}$ mice, which were equivalent. We also enumerated GATA3-positive Th2 cells in lymph nodes between naive WT and $Fbln1c^{-/-}$ mice by flow cytometry, and found that there were no differences (supplementary material, Figure S3).

As genetic deletion in $Fbln1c^{-/-}$ mice reduced chronic HDM-induced inflammation, the therapeutic effects of *Fbln1c*-targeted siRNA treatment were assessed. Treatment reduced the numbers of total leukocytes and of neutrophils, eosinophils and lymphocytes (Figure 5G) in BALF, and the levels of IL-5, IL-13, IL-33, TNF and CXCL1 (Figure 5H–L) in the lungs, as compared with scrambled siRNA treatment or vehicle after chronic HDM challenge. There were also fewer GATA3-positive Th2 cells in lymph nodes (Figure 5M; supplementary material, Figure S3) and lungs (Figure 5N; 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 extract or saline were collected. The levels of secretion from lymph node T cells from HDM-challenged WT mice of IL-5, IL-13 and TNF were all substantially increased as compared with saline-challenged controls (Figure 6A–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 than those from similarly challenged WT mice, although levels were still

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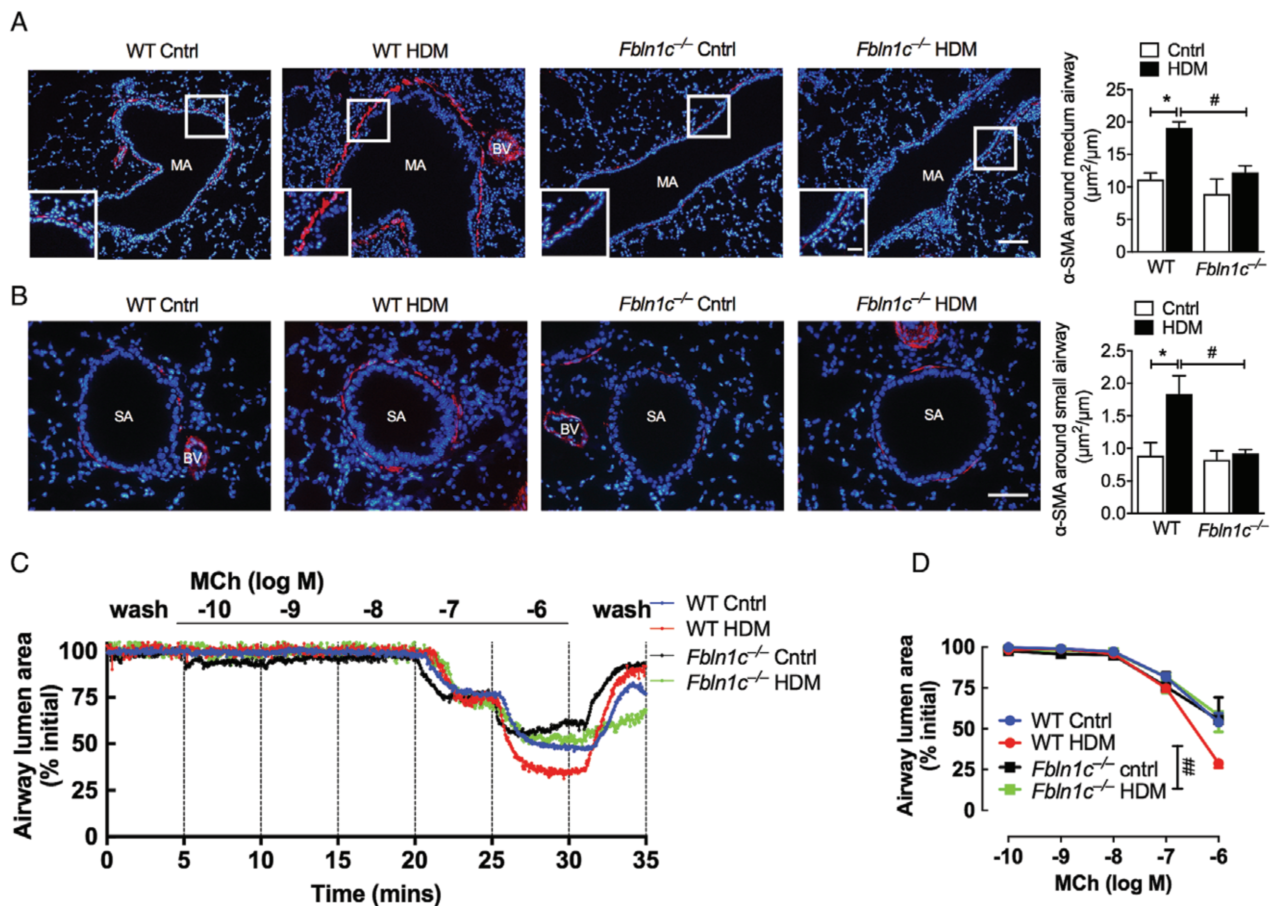

Figure 4. *Fbln1c*$^{-/-}$ mice are protected against increases in α-SMA-positive cell numbers around medium and small airways, and exaggerated airway contractility in HDM-induced experimental chronic asthma. (A and B) α-SMA (red) and nuclear (blue) staining around (A) medium and (B) small airways (left). Scale bars: 200 μm. Insets show expanded images 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. Results are mean ± SEM. $n = 6-8$ airways from $n = 5-8$ mice per group. $^*P < 0.05$ as compared with saline-challenged WT or *Fbln1c*$^{-/-}$ controls. $^{\#}P < 0.05$ as compared with HDM-challenged WT controls. Cntrl, control.

elevated as compared with saline-challenged *Fbln1c*$^{-/-}$ controls.

We next determined whether Fbln1c affected DC and/or Th2 cell activity and/or DC-induced Th2 responses by measuring the levels of IL-5 and IL-13 secreted from co-cultured DC and T cells restimulated with HDM extract. *Fbln1c*-targeted siRNA treatment affected both DC and Th2 cells and reduced IL-5 (Figure 6D) and IL-13 (Figure 6E) production as compared with cells from mice treated with scrambled siRNA. This occurred when *Fbln1c* siRNA-treated DCs or T cells were cultured with scrambled siRNA-treated T cells or DCs, respectively. The greatest effect was seen 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 in 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, so human Fbln1c and scrambled peptides were obtained as described previously [42]. Human bronchoepithelial A549 cells and minimally immortalized epithelium-derived basal (BCi-NS1.1) cells were cultured on plates coated with Fbln1c, coated with scrambled peptides, or uncoated and containing medium only, and RNA was extracted after 24 and 48 h. *CXCL1* mRNA levels were significantly increased in cells exposed to Fbln1c peptide as compared with culture medium, and there were non-statistically significant trends towards increases as compared with scrambled peptide treatment ($P < 0.06$ and $P < 0.08$, respectively; Figure 6F, G).

Discussion

Increased production and deposition of ECM proteins, the resultant airway remodelling and AHR and pulmonary inflammation are key pathological features of asthma. Our previous studies have shown that Fbln1 levels are increased in BALF and ASM cells from asthma

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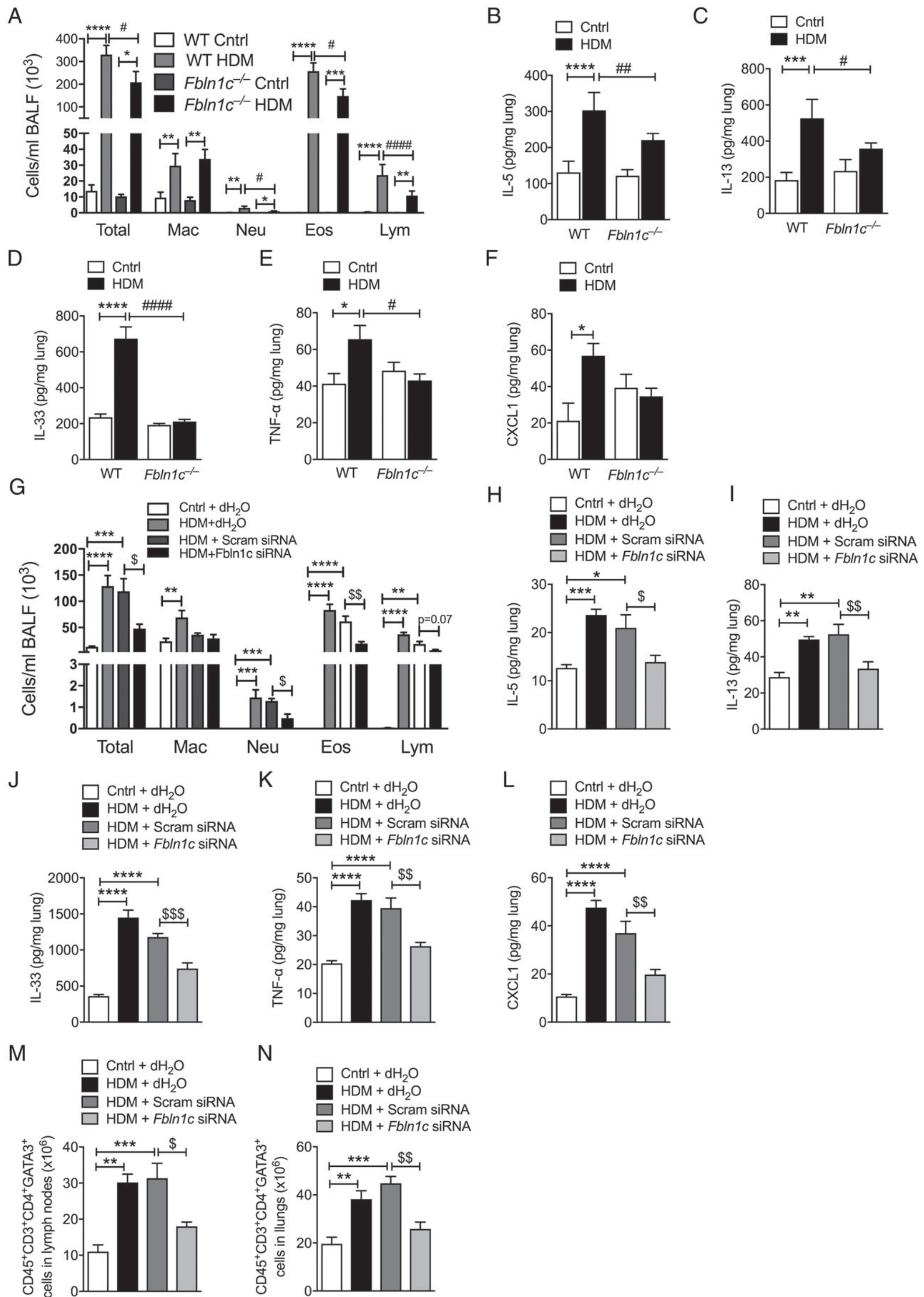

Figure 5. Legend on next page

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patients as compared with 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 the levels of Fbln1, Fbln1c and collagen are increased in an HDM-induced mouse model of experimental chronic asthma. Genetic deletion of *Fbln1c* 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, which probably also contributed to the protection against AHR. We also showed that Fbln1c is involved in allergic airway inflammation, because deletion of *Fbln1c* in mice resulted in less influx of leukocytes, including neutrophils, eosinophils, and lymphocytes, into the airways than in 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 as compared with WT mice. Deletion 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 an Fbln1c peptide had increased *CXCL1* mRNA levels as compared with medium-exposed controls, and showed a trend towards an increase as compared with 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 the levels of Col1a1, Fbln1 and Fbln1c in whole lungs. Collagen deposition around the small airways started to occur after 21 days, and Col1a1 levels were increased after 28 days of HDM challenge. Fbln1 and Fbln1c levels 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 stabilize 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 stabilizing ECM protein deposition by binding to multiple ECM targets to maintain the structure of the airways and lungs [22]. We showed that increases in Fn and Postn levels 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 organize 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 of Fn [46], and promotes cell adhesion and has motility-suppressive effects on Fn-coated substrates [47]. Increased levels of Fn 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 in the lungs of asthma patients [48]. Furthermore, both Fn and Postn bind to collagen and Fbln1, and Postn and Tnc co-localize in skin keratinocytes [19,49,50]. The reduction in Fn levels in whole lungs of *Fbln1c*$^{-/-}$ mice after HDM challenge probably reflects the importance of Fbln1c in stabilizing Fn fibres in the ECM matrix. Peribronchial Fn levels were reduced after HDM challenge in *Fbln1c*$^{-/-}$ mice, and the amount of Fn was reduced in the whole lungs even as compared with saline-challenged controls. In WT mice, we found decreased levels of Tnc in whole lungs, but increased levels around airways after HDM challenge. Our previous study also demonstrated that the levels of ECM proteins were different between airways and parenchyma in *Fbln1c*$^{-/-}$ mice with experimental chronic obstructive pulmonary disease [22]. The observation of different changes in the airways and in whole lungs suggests that ECM proteins may be produced differently in different regions, such as the airways and parenchyma. Indeed, fibroblasts/myofibroblasts, ASM cells and epithelial cells are major sources of Fn around airways [21,42], but fibroblasts and alveolar epithelial cells are the primary sources in the parenchyma [51].

We previously showed that fibroblasts [42], bronchoepithelial cells [22] and ASM cells [21] all produce Fbln1 in the airways and lungs. In this study, we showed that Fbln1c regulates the proliferation of ASM cells and fibroblasts/myofibroblasts around the airways

Figure 5. Mice with genetic deletion of *Fbln1c* and therapeutic inhibition of Fbln1c are protected against pulmonary inflammation in HDM-induced experimental chronic asthma. WT and *Fbln1c*$^{-/-}$ mice received chronic HDM extract for 35 days. Controls received saline. (A) Differential inflammatory cell counts in BALF. (B–F) IL-5 (B), IL-13 (C), IL-33 (D), TNF (E) and CXCL1 (F) levels in whole lung homogenates measured by ELISA. Mice had HDM extract administered 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–L) IL-5 (H), IL-13 (I), IL-33 (J), TNF (K) and CXCL1 (L) levels in whole lung homogenates. (M and N) GATA3-positive Th2 cells from lymph nodes (M) and lungs (N) were enumerated by flow cytometry. Results are mean ± SEM. $n = 6$–8 mice per group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$ as compared with saline-treated or dH_2O-treated WT or *Fbln1c*$^{-/-}$ controls. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#\#}P < 0.0001$ as compared with HDM-treated WT controls. $^{\$}P < 0.05$ and $^{\$\$}P < 0.01$ as compared with HDM controls treated with scrambled siRNA. Cntrl, control; Eos, eosinophil; lym, lymphocyte; Mac, macrophage; Neu, neutrophil.

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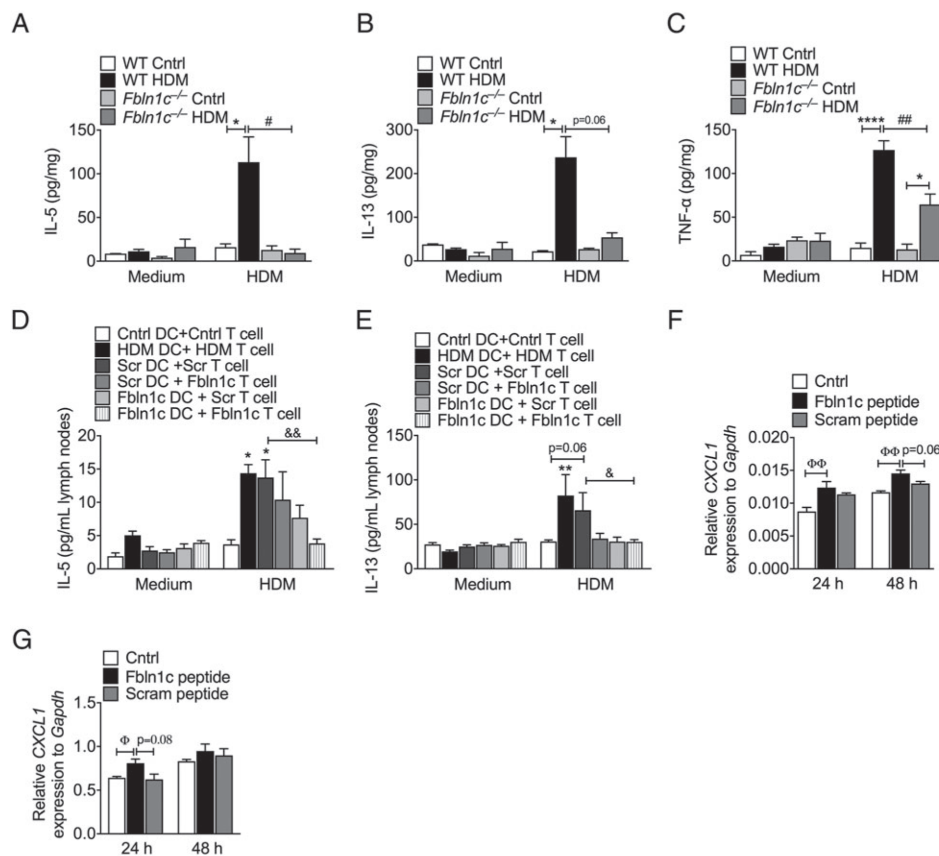

Figure 6. Genetic deletion of *Fbln1c* and therapeutic depletion of Fbln1c in mice protects against increased Th2 cytokine and chemokine production in lung-draining lymph nodes in HDM-induced experimental chronic asthma, and against Fbln1c peptide-induced CXCL1 production in a human epithelial cell line. Cells were isolated from the lymph nodes of WT and *Fbln1c*$^{-/-}$ mice and cultured with HDM extract, and (A) IL-5, (B) IL-13 and (C) TNF levels in supernatants were measured by ELISA. Mice were sensitized and challenged with HDM extract for 35 days to induce experimental asthma, and were treated with *Fbln1c*-targeted or scrambled siRNA. DCs and T cells were isolated from lymph nodes and restimulated with HDM extract, and IL-5 (D) and IL-13 (E) 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 on plates coated with Fbln1c peptide, coated with scrambled peptide, or uncoated and containing medium only, and *CXCL1* mRNA levels were measured by reverse transcription qPCR. $n = 6$. Results are mean ± SEM. $^{*}P < 0.05$ and $^{****}P < 0.0001$ as compared with saline-challenged WT or *Fbln1c*$^{-/-}$ controls with HDM restimulation. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ as compared with HDM-challenged WT mice with HDM restimulation. $^{\&}P < 0.05$ and $^{\&\&}P < 0.01$ as compared with HDM-challenged mice with scrambled siRNA. $^{\emptyset}P < 0.05$ and $^{\emptyset\emptyset}P < 0.01$ as compared with culture medium control. Cntrl, control.

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 suggest that the numbers of either or both of these cell types were reduced. In this study, we also showed 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 in asthma patients is reduced after targeting of *Fbln1c* mRNA [21]. Fibroblasts/myofibroblasts are major cellular sources of ECM proteins [57], and their numbers are increased in the lung tissues of asthmatic children as compared with healthy controls [58]. Myofibroblasts, in particular, are major producers of type I and III collagen [59]. Thus, potential decreases in the numbers of ASM cells and/or myofibroblasts in our study may be the cause of the reduced collagen deposition in *Fbln1c*$^{-/-}$ mice.

MUCs play critical roles in mucus secretion and AHR in asthma, and previous studies have shown that genetic deletion of *MUC5AC* prevents AAD-induced AHR in mice [60]. We showed that *Fbln1c*$^{-/-}$ mice had reduced MUC5AC but not MUC5B levels in airways as compared with WT controls. Although increased mucus secretion in the lungs occurs in asthma [61], we found that the numbers of mucus-secreting cells were not different between *Fbln1c*$^{-/-}$ and 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

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involved in airway remodelling [62,63], whereas the chemokine CXCL1 contributes to the inflammatory process in asthma [64]. WT mice challenged with HDM extract showed significant increases in the levels of asthma-related cytokines (IL-5, IL-13, IL-33, and TNF) in their lungs and lung-draining lymph nodes. However, deletion of *Fbln1c* in mice protected against increases in the levels of these factors in chronic experimental asthma. We showed that there were fewer GATA3-positive T cells in the lymph nodes and lungs of mice treated with *Fbln1c* siRNA than in the lymph nodes and lungs of mice treated with scrambled siRNA. We also showed that 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 have demonstrated 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 because of 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 in lung tissue than WT mice. Although the exact mechanism remains unclear, we showed that Fbln1c peptide increased *CXCL1* mRNA levels in two human epithelial cell lines as compared with vehicle, but showed only non-statistically significant trends to cause increases as compared with scrambled peptide-treated controls. We interpret these data as indicating 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 stabilizes deposition of ECM proteins, including Fn and Postn, and regulates collagen levels around the airways and in lungs. This results in airway remodelling and AHR in HDM-induced chronic experimental asthma. Fbln1c is required for increases in the numbers of 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

The authors contributed in the following way: GL, AGJ, JKB, PMH: participated in design of the study; GL: performed all *in vivo* and most *in vitro* experiments; MAC, WSA: generated $Fbln1c^{-/-}$ mice; PMN, TJH: assisted with mouse experiments; CD, JEB, CLG: helped to perform mouse airway contractility studies; ACH, PAW: assisted with experiments using human minimally immortalized epithelium-derived basal (BCi-NS1.1) cells; ACB, HT, PSF: assisted with T-cell isolation and flow cytometry; GL, MAC, QG, NGH, JCH, BGO, DAK, JKB, PMH: contributed to preparation and editing of the manuscript for intellectual content. All authors read and approved the final manuscript (with the exception of WSA, who passed away before the final version was completed).

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SUPPLEMENTARY MATERIAL ONLINE

Supplementary materials and methods

Supplementary figure legends

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

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