

Defective Fibrillar Collagen Organization by Fibroblasts Contributes to Airway Remodeling in Asthma

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Abstract

Rationale: Histologic stains have been used as the gold standard to visualize extracellular matrix (ECM) changes associated with airway remodeling in asthma, yet they provide no information on the biochemical and structural characteristics of the ECM, which are vital to understanding alterations in tissue function.

Objectives: To demonstrate the use of nonlinear optical microscopy (NLOM) and texture analysis algorithms to image fibrillar collagen (second harmonic generation) and elastin (two-photon excited autofluorescence), to obtain biochemical and structural information on the remodeled ECM environment in asthma.

Methods: Nontransplantable donor lungs from donors with asthma ($n = 13$) and control ($n = 12$) donors were used for the assessment of airway collagen and elastin fibers by NLOM, and extraction of lung fibroblasts for *in vitro* experiments.

Measurements and Main Results: Fibrillar collagen is not only increased but also highly disorganized and fragmented within large and small asthmatic airways compared with control subjects, using NLOM imaging. Furthermore, such structural alterations are present in pediatric and adult donors with asthma, irrespective of fatal disease. *In vitro* studies demonstrated that asthmatic airway fibroblasts are deficient in their packaging of fibrillar collagen-I and express less decorin, important for collagen fibril packaging. Packaging of collagen fibrils was found to be more disorganized in asthmatic airways compared with control subjects, using transmission electron microscopy.

Conclusions: NLOM imaging enabled the structural assessment of the ECM, and the data suggest that airway remodeling in asthma involves the progressive accumulation of disorganized fibrillar collagen by airway fibroblasts. This study highlights the future potential clinical application of NLOM to assess airway remodeling *in vivo*.

Keywords: asthma; airway remodeling; collagen; fibroblasts; nonlinear optical microscopy

Asthma is characterized by chronic airway inflammation (which forms the target of all asthma therapeutics) and airway remodeling that involves all tissues of the airway wall including the epithelium,

basement membrane, lamina propria, smooth muscle, and vascular structures (1), for which there are no therapeutic treatments. Airway remodeling was first described in cases of fatal asthma by

Huber and Koessler (2). Since then, airway remodeling has been documented for all stages of asthma severity and has been linked to reduced lung function, airways hyperresponsiveness, and a

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At a Glance Commentary

Scientific Knowledge on the

Subject: Histologic stains have been used as the gold standard to visualize morphologic and extracellular matrix changes associated with airway remodeling in asthma. Using such stains, it has been shown that collagen is greatly accumulated within asthmatic airways compared with control subjects. However, histologic stains provide no information on the biochemical and structural characteristics of extracellular matrix fibers, which are vital to understanding disease pathology.

What This Study Adds to the

Field: This study demonstrates the use of nonlinear optical microscopy to assess the biochemical and structural characteristics of collagen and elastin fibers in asthmatic airways. Importantly, the data demonstrate that accumulation of disorganized fibrillar collagen is an early feature of airway remodeling being present in pediatric and adult donors, irrespective of fatal disease. Furthermore, *in vitro* studies identified that asthmatic-derived airway fibroblasts are defective at collagen repair, and express less decorin, which is important for collagen formation, providing a potential mechanism for disorganized fibrillar collagen in asthma. Clinical studies demonstrate the application of nonlinear optical microscopy for use with endoscopy techniques to assess extracellular matrix remodeling. This opens the opportunity for future use of nonlinear optical microscopy and texture analysis to assess and monitor airway remodeling longitudinally and in response to targeted therapeutic treatments.

greater use of asthma medications (3–6). It was previously proposed that chronic inflammation involving mast cells, eosinophils, neutrophils, and CD4⁺ T cells (7–9) leads to airway remodeling over the lifetime of an individual with asthma. However, recent studies have now shown that airway remodeling is not present at birth (10), but features of

remodeling are present early (11, 12), even by the age of 2–4 years and often before atopic inflammation is observed or a clinical diagnosis of asthma is made (13).

Since 1927, airway remodeling has been assessed using histologic chemical stains, such as Verhoeff-van Gieson or Masson trichrome, to visualize alterations in cell structures and tissue components. Using such stains, it has been shown that collagen is greatly accumulated within asthmatic airways compared with control subjects (12). Fibrillar collagens are the predominant collagen in the human body, and the concentration and distribution of collagen fibers is essential for normal tissue function (14). Fibrillar collagens are also tightly packaged with other extracellular matrix (ECM) molecules, such as elastin, enabling tissues to undergo repetitive strain and relaxation, such as during breathing (15). Alterations in the concentration and distribution of collagen and elastin fibers can therefore lead to loss of anatomic structure, compromised function, and tissue fibrosis (16, 17). However, clinically used histologic stains provide

no information on the biochemical and structural characteristics of ECM fibers, which is vital to understanding disease pathology.

Here we demonstrate the use of multimodal nonlinear optical microscopy (NLOM) to assess the biochemical and structural characteristics of collagen (second harmonic generation [SHG]) and elastin (two-photon excited autofluorescence) fibers (18, 19). Using texture and image analysis we provide new insight into the disorganization of fibrillar collagen fibers and fibrils in the remodeled airways of donors with asthma. Furthermore, our data demonstrate that asthmatic-derived airway fibroblasts are defective at collagen fiber organization, and express less decorin, which is essential for normal collagen fiber formation. This study expands the understanding of airway remodeling in asthma, and creates new opportunities for potential future clinical applications of NLOM to assess airway remodeling longitudinally, and in response to targeted therapeutic treatments. Some of these data have been previously reported in the form of abstracts (20, 21).

Table 1. Donor Demographics

	Group	
	Control Subjects	Subjects with Asthma
<i>n</i>	12	13
Age, mean (SD), yr	24.2 (13.7)	18.8 (8.7)
Sex, F, %	41.7	46.2
Height, mean (SD), cm	162.8 (19.8)	166.5 (10.2)
Weight, mean (SD), kg	70.3 (28.0)	63.6 (23.8)
Ethnicity/race, %		
White	83.4	84.6
Hispanic	8.3	15.4
Others	8.3	—
Medications, %		
Albuterol	—	76.9
Advair*	—	15.4
Singulair	—	7.7
Roxycodone*	—	7.7
Zyrtec	—	7.7
Cause of death, %		
Asphyxiation	—	15.4
Anoxia seizure	25.0	—
CNS tumor	8.3	—
CVA	16.7	—
FAE	—	61.5
Head trauma	50.0	23.1

Definition of abbreviations: CNS=central nervous system; CVA=cardiovascular attack; FAE=fatal asthma episode.

There was no statistical difference in age ($P=0.37$) or sex ($P=0.58$) between the two groups.

*Indicates medication combined with other.

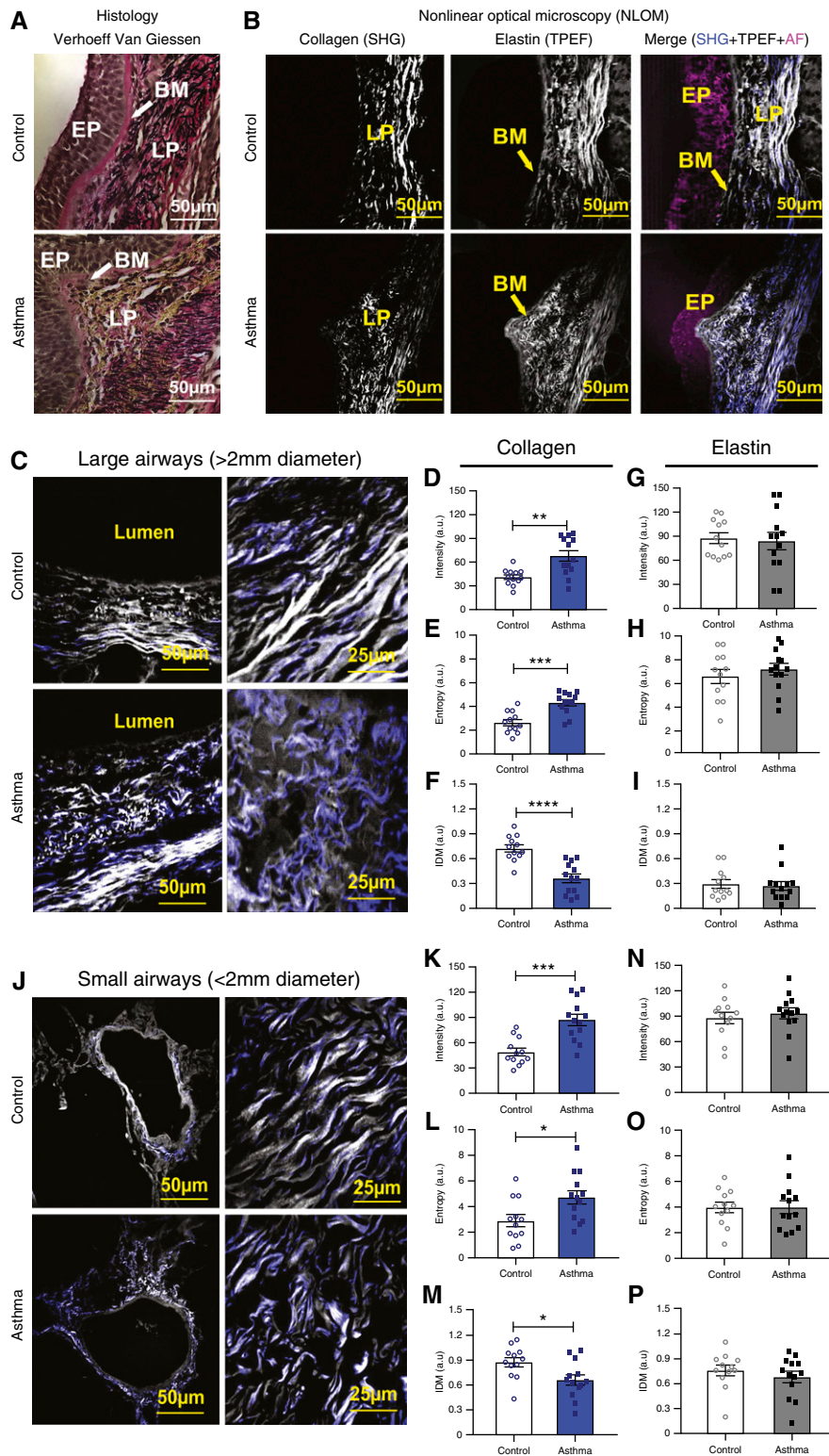


Figure 1. Nonlinear optical microscopy (NLOM) demonstrates fibrillar collagen is disorganized in asthmatic airways. (A and B) Control and asthmatic large airways visualized with Verhoeff-van Gieson stain (elastic fibers shown in black, collagen fibers shown in brown-yellow) (A) and label-free NLOM (B), where elastic fibers imaged with NLOM/two-photon excitation fluorescence (TPEF) are color coded in white, and collagen fibers imaged by NLOM/second harmonic generation (SHG) are color coded in blue (visualized by TPEF autofluorescence of the tissue color coded in magenta).

Methods

See the online supplement for full METHODS.

Patient Characteristics

Nontransplantable donor lungs from donors with asthma ($n = 13$) and control donors ($n = 12$) were donated for research, with informed consent from the next-of-kin through the International Institute for the Advancement of Medicine. The study was approved by the Providence Health Care Research Ethics Board (H13-02173) at the University of British Columbia. The demographic and clinical characteristics of all donors are presented in Table 1. There was no statistical difference in the asthmatic and control group with regards to age and sex. In the asthmatic group, 7 out of 13 died from a fatal asthma episode.

Sampling of Lung Tissue

Using surgical clamps airway and parenchymal tissue were blunt dissected for extraction of tissue for transmission electron microscopy analysis and fibroblast cultures as previously described (22, 23). The lung was then inflated, frozen, and cut into contiguous 2-cm-thick slices (transaxial plane) as previously described (24). A uniform random sampling method was applied to select sampling sites using a line grid (225 mm²) that was superimposed onto the photographs of each lung slice. Sample locations (line intersection) falling on connective tissue or large airways and blood vessels were also excluded from the sampling. Three 15 × 20-mm cylindrical tissue cores were taken for formalin fixation and paraffin embedding. Tissue sections of 5 μm were stained with Verhoeff-van Gieson stain and digitally scanned (ScanScope XT slide scanner; Aperio Technologies), or stained with hematoxylin and used for NLOM. Airways were segregated into small (<2 mm diameter) or large (>2 mm diameter) airways.

Nonlinear Optical Microscopy

Lung tissue samples were imaged using an in-house built multimodal femtosecond NLOM (18, 19), capable of acquiring colocalized two-photon excited autofluorescence and SHG images

simultaneously. For each donor, four airways in total were imaged, from which three randomly selected regions of interest were analyzed.

Texture Analysis

Image background correction, intensity normalization, and texture analysis were performed using Fiji analysis toolbox and plugins (25). Entropy (a measure of the degree of disorder of the structures within an image) and inverse difference moment (a metric related to the homogeneity of the structures within the image), first defined by Haralick and colleagues (26), were calculated using a gray level cooccurrence matrix, in a custom-built texture analysis toolkit in MATLAB (18). Fibers were identified using a method for unbiased detection of curvilinear structures based on ridge detection (27). By locating the first and second directional derivatives of the image for the extraction of the line points, it was possible to further calculate fiber thickness, overlap, and spacing. The fiber directionality analysis was performed using Fourier spectrum analysis (28).

Transmission Electron Microscopy

Images were obtained using a Philips/FEI Tecnai 12 transmission electron microscopy at $\times 37,000$ magnification. Cross-sectional images of collagen fibers were used to calculate the orientation of collagen fibrils using the index of circularity (18) in MATLAB.

Fibroblast Experiments

Primary airway and parenchymal fibroblasts were obtained using an outgrowth technique as previously described (22) and used at passages 1–2. In each experiment, 50,000 cells were treated with or without 10 ng/ml TGF- β 1 (transforming growth factor- β 1) and assessed for collagen remodeling and contraction using a free-floating three-

dimensional collagen I gel contraction assay on which cells were seeded and allowed to migrate for 72 hours (29, 30), or cell stiffness measured using optical magnetic twisting cytometry (31). Following functional experiments collagen gels were used for NLOM imaging, protein, or mRNA quantification. The number of fibroblasts present in each collagen gel was assessed by counting nuclei stained with DAPI using a Fiji customized macro (32). All cell culture experiments were run in triplicate.

Statistical Analysis

A D'Agostino-Pearson and Shapiro-Wilk normality test was used to assess the data. For nonparametric data two-tailed Mann-Whitney *U* tests were performed using GraphPad Prism version 6.0, linear mixed-effect models (nlme package), and power calculations (bootstrap) were performed using the statistical software R3.3.1 (RStudio). Data are shown as mean $\pm$ SEM, $P < 0.05$ was considered significant, and all two-tailed Mann-Whitney *U* tests had greater than 0.90 power.

Results

NLOM Demonstrates Fibrillar Collagen Is Disorganized in Asthmatic Airways

Compared with the clinically used Verhoeff-van Gieson stain (collagen fibers, yellow; elastic fibers, black) (Figure 1A) we demonstrate the capacity for high-resolution NLOM (Figure 1B) to image fibrillar collagen using SHG and elastin using two-photon excited autofluorescence in control and asthmatic airways.

The ability to resolve fibrillar collagen and elastin fibers with NLOM enabled the use of texture analysis to assess changes in fiber organization and integrity. For the

analysis, conducting airways were divided into large (>2 mm in diameter, Figure 1C) and small (<2 mm in diameter, Figure 1J) airways. There was no statistical difference in the mean small airway diameter (control donors, 704.1 ± 83.1 μ m vs. donors with asthma, 753.1 ± 117.2 μ m), or large airway diameter (control donors, 5.81 ± 0.98 mm vs. donors with asthma, 5.3 ± 1.27 mm) between groups.

We found increased levels of fibrillar collagen (intensity) in the lamina propria of large (Figure 1D) and small (Figure 1K) airways of patients with asthma compared with control subjects. Using texture analysis we were able to demonstrate for the first time that fibrillar collagen fibers within the lamina propria of asthmatic large and small airways are extensively disorganized having greater entropy (degree of disorder of the structures within an image) (Figures 1E and 1L), and are less uniform with a decreased inverse difference moment (homogeneity of structures within an image) (Figures 1F and 1M) compared with control airways. It is well documented that smooth-muscle mass increases contribute to ECM production and airway closure in asthma (33, 34). In contrast to the laminar propria, we found collagen within the smooth muscle region to be increased but not disorganized (see Figures E1A–E1D in the online supplement). We observed no alterations in the intensity or organization of elastin within the lamina propria (Figures 1G–1I and 1N–1P) or smooth muscle (see Figures E1E–E1G) region of asthmatic and control airways.

Fibrillar Collagen Is Disorganized in Asthmatic Airways Irrespective of Age, Sex, and Fatal Asthma

Importantly, in subjects with asthma collagen fibers were found to be increased (intensity) and extensively disorganized (entropy) within the lamina propria of large and small airways irrespective of age

Figure 1. (Continued). (C and J) Representative low- and high-magnification images of the SHG and TPEF signal from large airways (C) and small airways (J) of control donors and donors with asthma. (D–F and G–I) Graphical analysis of collagen (SHG signal) (D–F) and elastin (TPEF signal) (G–I) textural analysis features intensity, entropy, and inverse difference moment (IDM) imaged in the lamina propria of large airways from donors with asthma and control donors. (K–M and N–P) Graphical analysis of collagen (SHG signal) (K–M) and elastin (TPEF signal) (N–P) textural analysis features intensity, entropy, and IDM imaged in the lamina propria of small airways from donors with asthma and control donors. Bar graphs represent the mean, and error bars denote SEM. Circles and squares depict data from 12 biologically independent control donors (total of 48 airways, average of four/donor, three regions of interest/airway) and 13 biologically independent donors with asthma (total of 52 airways, average of four/donor, three regions of interest/airway). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$. AF = airway fibroblasts; BM = basement membrane; EP = airway epithelial cells; LP = lamina propria.

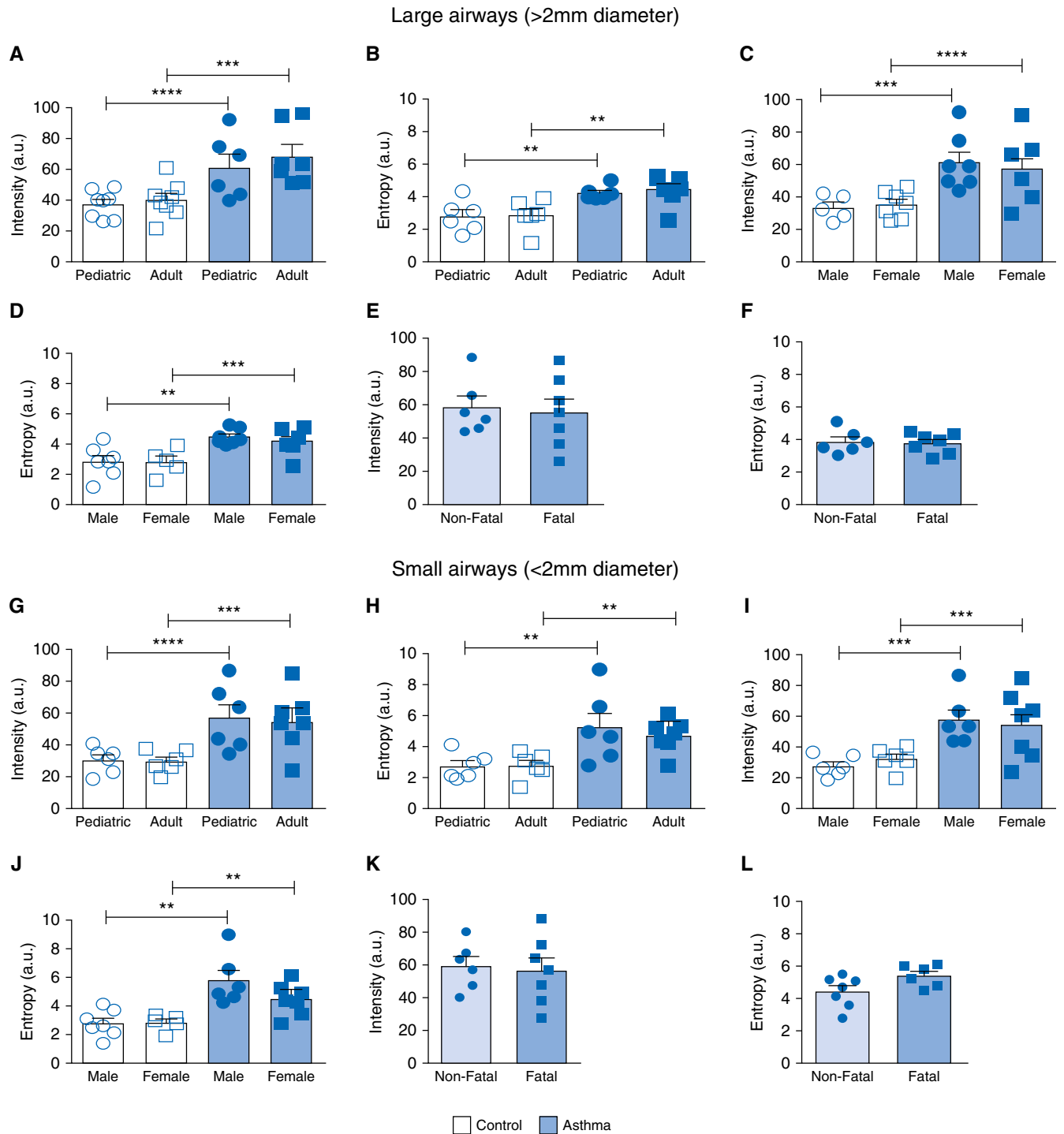


Figure 2. Fibrillar collagen is disorganized in asthmatic airways irrespective of age, sex, and fatal asthma. (A–L) Intensity and entropy features were obtained using texture analysis from nonlinear optical microscopy/second harmonic generation images of fibrillar collagen within the lamina propria of control (white bars) and asthmatic (blue bars) large (A–F) and small (G–L) airways. Subjects were divided by age (pediatric [<16 yr of age] or adult [>18 yr of age]), sex (male or female), and disease severity (fatal asthma episode [fatal] or nonfatal). (A, C, E, G, I, and K) The intensity of the nonlinear optical microscopy/second harmonic generation signal for fibrillar collagen is shown for large and small airways by pediatric and adult (A and G), male and female donors (C and I), and fatal and nonfatal cases of asthma (E and K). (B, D, F, H, J, and L) The entropy of the nonlinear optical microscopy/second harmonic generation for fibrillar collagen is shown for large and small airways by pediatric and adult (B and H), male and female donors (D and J), and fatal and nonfatal cases of asthma (F and L). Bar graphs represent the means, and error bars denote SEM. Circles and squares depict data from 12 biologically independent control donors (total of 48 airways, average of four/donor, three regions of interest/airway) and 13 biologically independent donors with asthma (total of 52 airways, average of four/donor, three regions of interest/airway). ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

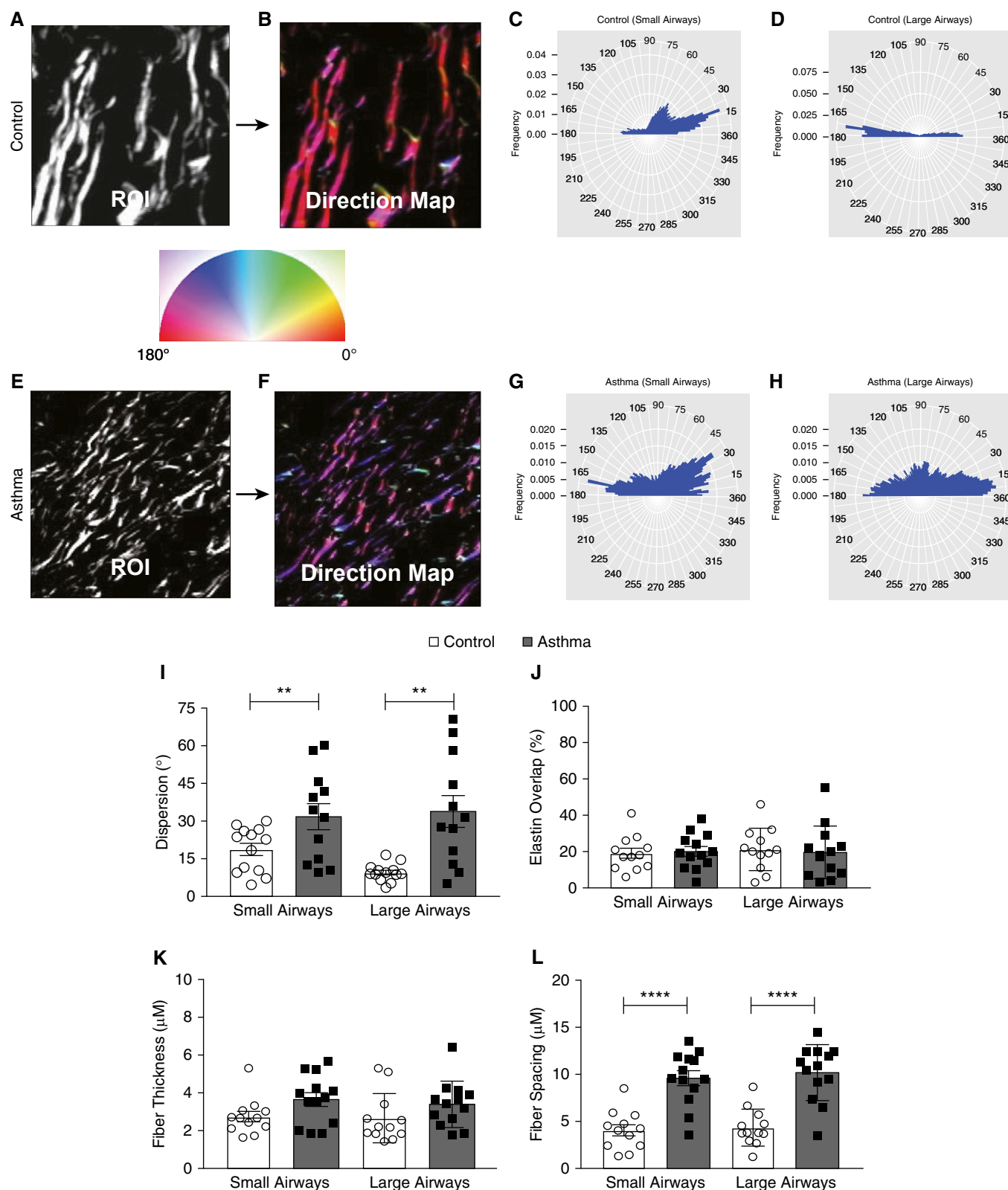


Figure 3. Structural characteristic of fibrillar collagen in remodeled airways. (A, B, E, and F) Representative regions of interest (ROIs) from nonlinear optical microscopy/second harmonic generation images of the lamina propria of small and large airways showing fibrillar collagen fibers and its color-coded orientation from control donors (A and B) and donors with asthma (E and F). (C, D, G, and H) Representative histograms show the preferred direction of collagen fibers in small airways from control donors (C) and donors with asthma (G) and in large airways from control donors (D) and donors with

(Figures 2A, 2B, 2G, and 2H), sex (Figures 2C, 2D, 2I, and 2J), or fatal asthma (Figures 2E, 2F, 2K, and 2L). Multiple-regression analysis further verified this finding with only asthma being significantly associated with increased collagen intensity (estimate, -27.79 ; $P = 0.0015$) and entropy (estimate, -1.63 ; $P = 0.0002$), and not age, sex, or fatal disease, indicating collagen disorganization occurs early in the disease process, and is present irrespective of fatal disease.

Structural Characteristic of Fibrillar Collagen in Remodeled Airways

We analyzed the spatial arrangement of fibrillar collagen fibers in control (Figures 3A and 3B) and asthmatic airways (Figures 3E and 3F) using directionality Fourier transform analysis. Histograms of the directionality dispersion analysis of control airways demonstrates that fibrillar collagen fibers within large airways (Figure 3D) had a lower dispersion compared with small airways (Figure 3C), which is to be expected because of the known banding of fibrillar collagen within large airways. However, fibrillar collagen fibers in both large (Figure 3H) and small (Figure 3G) asthmatic airways had a greater directionality dispersion when compared with control airways (Figure 3I). These data demonstrate the fibrillar collagen fibers within the lamina propria of asthmatic airways are more fragmented and do not present a preferred orientation compared with control airways. We found no changes in elastin fiber directionality dispersion with asthmatic airways compared with controls (see Figure E2). Next, we assessed the overlap of fibrillar collagen and elastic fibers, and this remained consistent with 20% overlap in both control and asthmatic large and small airways (Figure 3J).

We also found no significant difference in collagen fiber thickness between donors with asthma and control donors (Figure 3K). We did find that the spacing between fibrillar collagen fibers was significantly greater in asthmatic airways compared with controls (Figure 3L).

Asthmatic-derived Airway Fibroblasts Are Defective at Fibrillar Collagen I Formation

To determine what cells were present within the disorganized fibrillar collagen of asthmatic airways, SHG imaging was combined with confocal imaging. Compared with the banding of fibrillar collagen in the lamina propria of control airways (Figure 4A), fibrillar collagen was highly irregular within the lamina propria of asthmatic airways (Figure 4B, purple), and these regions were associated with populations of mesenchymal cells positive for F-actin (green).

To determine if asthmatic airway mesenchymal fibroblasts were responsible for alterations in fibrillar collagen structure, primary airway and parenchymal mesenchymal fibroblasts were isolated from the lungs of the donors with asthma and control donors. We used a free-floating hydrolyzed rat-tail collagen I gel contraction assay, on which fibroblasts were seeded and allowed to migrate through the gel for 72 hours, to assess the ability of cells to repair and remodel fibrillar collagen I (29). We found that asthmatic-derived airway fibroblasts were unable to remodel and contract hydrolyzed collagen I to the same extent as control-derived airway fibroblasts (Figures 4C and 4D). This defect was specific to airway fibroblasts, because parenchymal fibroblasts derived from the same donors with asthma were not defective compared with control subjects (Figures 4C and 4E). The profibrotic cytokine TGF- β 1 is a well-known activator of fibroblasts during repair (35); however, treatment with 10 ng/ml TGF- β 1 for 72 hours or increasing the time for contraction to 144 hours did not alter the response of asthmatic airway fibroblasts (see Figure E3A).

To determine if defective collagen I gel contraction by asthmatic-derived airway fibroblasts was due to defective collagen fiber formation, we analyzed the SHG signal of fibrillar collagen I using NLOM-SHG (Figure 4F). Collagen I gels seeded with

asthmatic-derived airway fibroblasts had a significantly lower signal intensity (Figure 4G) and greater disorganization of fibrillar collagen I (entropy, Figure 4H) compared with control-derived airway fibroblasts, in line with the *ex vivo* imaging data. We found no differences in the NLOM-SHG signal for fibrillar collagen intensity or entropy, when comparing parenchymal fibroblasts derived from the same donors (Figures 4I and 4J).

Asthmatic-derived Airway Fibroblasts Have a Normal Functional Cytoskeleton

When we imaged (Figure 5A) and assessed the interaction of airway fibroblasts with fibrillar collagen, we found no difference between asthmatic and control airway fibroblasts for the numbers of cells (Figure 5B), the distance between cells (Figure 5C), or cell stiffness (G^1 [Pa/nm]) (Figure 5D) at baseline or in response to TGF- β 1 treatment (see Figure E3B). We also found no differences between asthmatic and nonasthmatic fibroblasts in the cytoskeletal protein expression of F-actin (Figure 5E), nonmuscle myosin IIB (Figure 5F), or β -tubulin (Figure 5G), which are known to be involved in the formation of lamellipodia and stress fibers, two distinct actin assembly mechanisms used by mesenchymal cells to repair and contract fibrillar collagen (30).

Asthmatic-derived Airway Fibroblasts Are Defective at Packaging Fibrillar Collagen I

We found no differences between asthmatic and control airway fibroblasts in their ability to express collagen I α 1 (Figure 6A) or lysyl oxidase (Figure 6B), lysyl oxidase like enzymes 1 and 2 (see Figures E4A and E4B) essential enzymes for the cross-linking of collagen fibers. However, we did find decreased expression of Decorin, a small leucine-rich proteoglycan, required for the normal spacing of collagen fibrils within fibers, in collagen I gels seeded with asthmatic fibroblasts compared with controls (Figure 6C). Using the

Figure 3. (Continued). asthma (H). (I) Graphical analysis of the dispersion angle for control donors (white bars) and donors with asthma (gray bars) in small and large airways. (J–L) Fibrillar collagen fibers were also assessed for percentage elastin–collagen overlap (J), collagen fiber thickness (K), and fiber spacing (L), in both small and large airways from control donors (white bars) and donors with asthma (gray bars). Bar graphs represent the means, and error bars denote SEM. Circles and squares depict data from 12 biologically independent control donors (total of 48 airways, average of four/donor, three ROIs/airway) and 13 biologically independent donors with asthma (total of 52 airways, average of four/donor, three ROIs/airway). ** $P < 0.01$ and **** $P < 0.0001$.

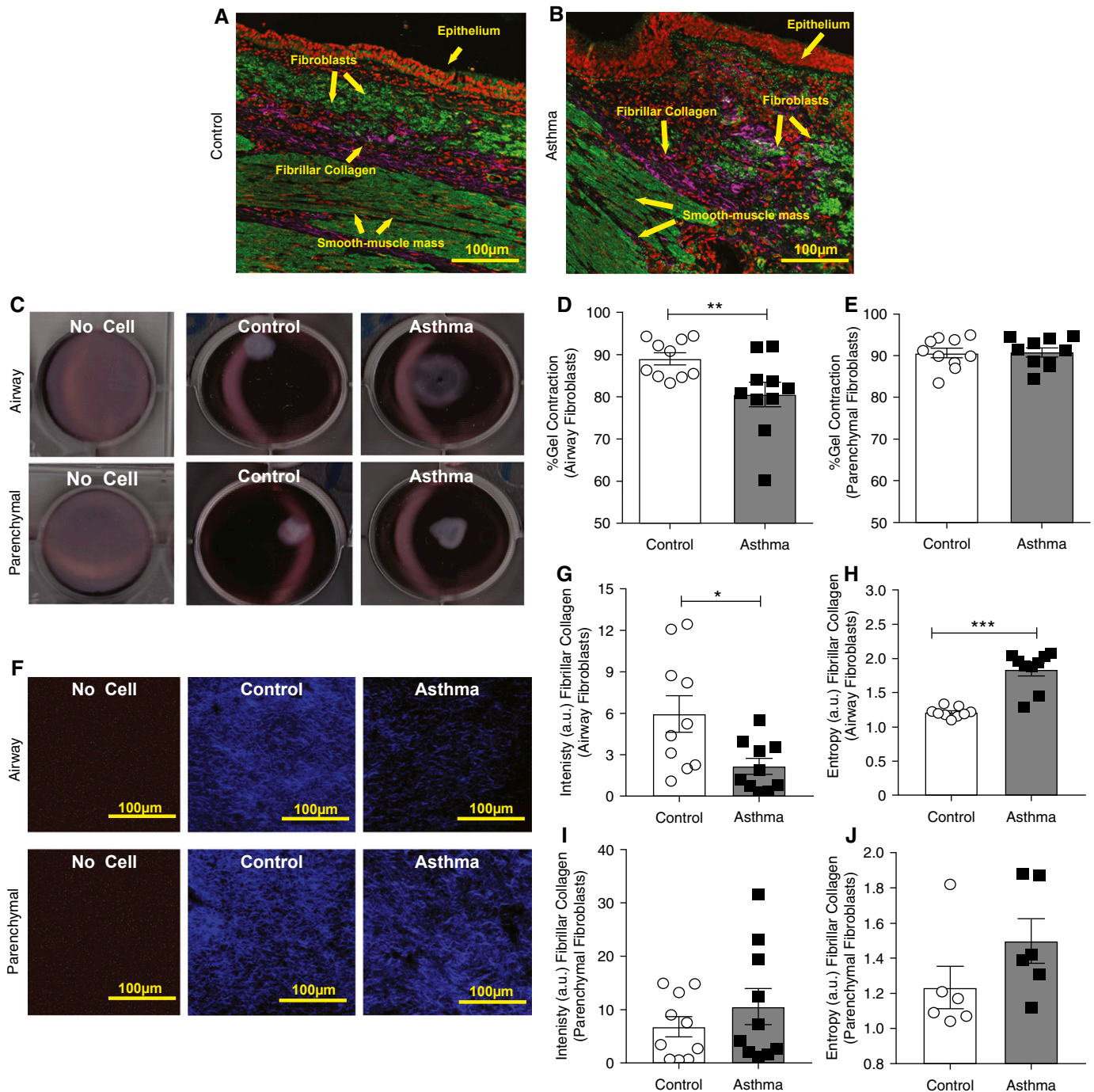


Figure 4. Asthmatic-derived airway fibroblasts are defective at fibrillar collagen I formation. (A and B) Representative confocal and nonlinear optical microscopy/second harmonic generation images, comparing the large airway wall from a control donor (A) and a donor with asthma (B). Nuclei are shown in red (propidium iodide), F-actin is shown in green (fluorescein isothiocyanate), and second harmonic generation signal for fibrillar collagen is shown in purple. Primary airway and parenchymal fibroblasts from control donors (white bars) and donors with asthma (gray bars) were seeded (40,000 cells) in rat-tail collagen I gels in triplicate and allowed to contract for 72 hours. (C) Representative light microscopy images of collagen gels at 72 hours. (D and E) Percentage gel area contraction of airway (D) and parenchymal fibroblasts (E). (F) Representative images of nonlinear optical microscopy/second harmonic generation signal from collagen I gels. (G–J) Average intensity (G and I) and entropy analysis (H and J) of fibrillar collagen I from airway (G and H) and parenchymal fibroblast-seeded collagen I gels (I and J). Bar graphs represent the means, and error bars denote SEM for 10 biologically independent control donors and donors with asthma. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

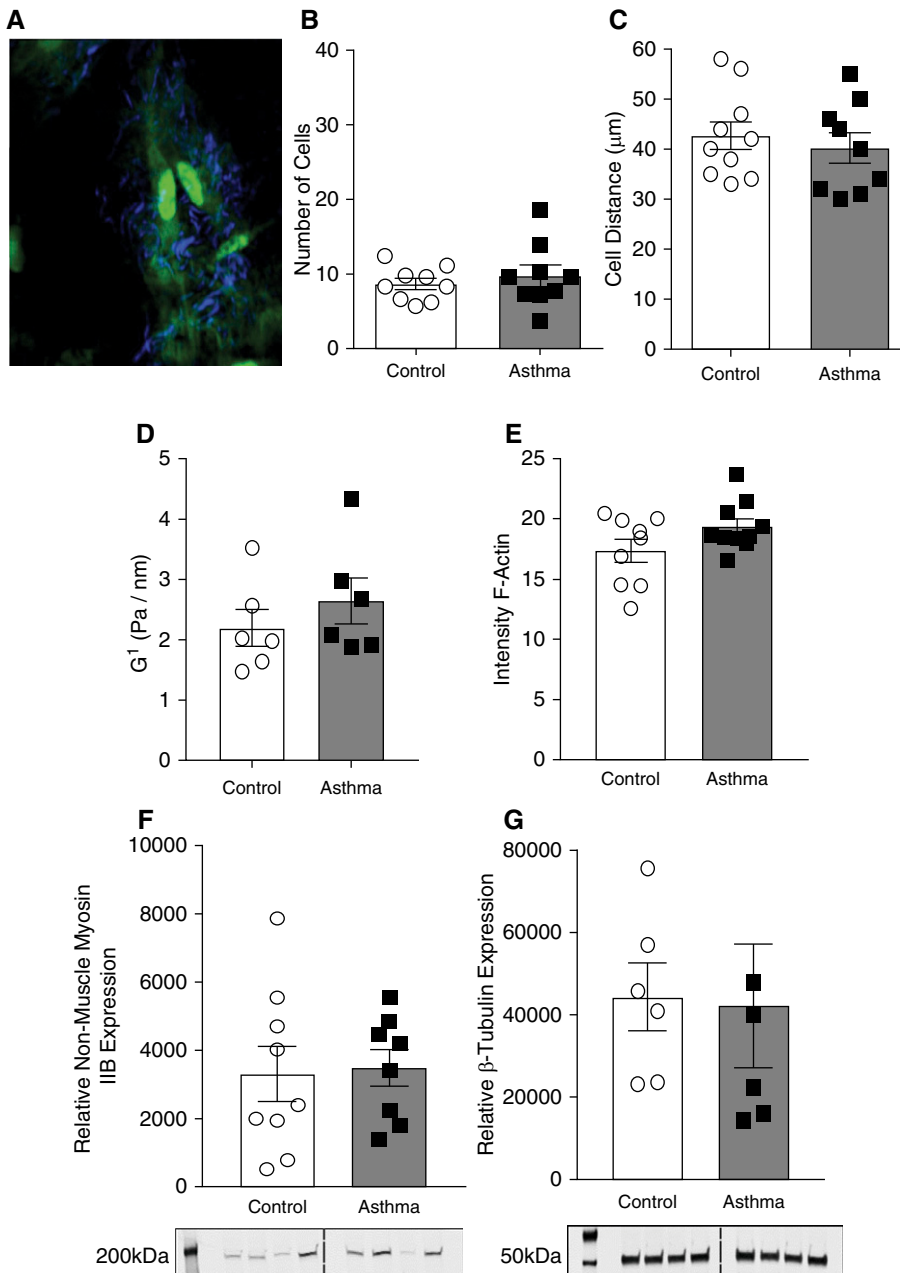


Figure 5. Asthmatic-derived airway fibroblasts have a normal functional cytoskeleton. (A) High-resolution representative image showing fibroblasts (stained for F-actin in green) interacting with fibrillar collagen I (second harmonic generation signal in blue). (B) Average number of airway fibroblasts per gel. (C) Average distance between neighboring airway fibroblasts in collagen I gels measured in μm . (D and E) Airway fibroblast cell stiffness ($n=6$ per group) (D) and intensity of F-actin (E). Primary airway fibroblasts from control subjects and subjects with asthma were seeded on collagen I-coated tissue culture plates and grown to confluence. (F and G) Relative protein expression of nonmuscle myosin II B (F) and β -tubulin ($n=8-5$ per group) (G). Bar graphs represent the means, and error bars denote SEM for control donors (white bars) and donors with asthma (gray bars). $P < 0.05$ was considered statistically significant.

ultraresolution of transmission electron microscopy, we demonstrate that compared with control airways (Figures 6D and 6E), asthmatic airways have disorganized

collagen fibril structures within collagen fibers (Figures 6F and 6G), quantified by an increased circularity score (Figure 6H) and index of dispersion (Figure 6I).

Discussion

For nearly a century, histologic stains have been used as the gold standard to visualize morphologic and ECM changes associated with airway remodeling in asthma. Here we demonstrate the application of high-resolution NLOM and texture analysis to obtain for the first time biochemical and structural information on the remodeled ECM environment in asthma. Our detailed study using human-derived tissues and primary cells demonstrates elevated levels of disorganized and fragmented fibrillar collagen fibers in asthmatic airways, and that defective decorin production by airway fibroblasts may be responsible for this defective fibrillar collagen formation. These findings have important implications for airway remodeling in asthma because disorganized collagen can profoundly affect the response of cells within the tissue microenvironment, and thus provides a potential new therapeutic target.

Airway remodeling has been documented for all stages of asthma severity and has been linked to reduced lung function, airways hyperresponsiveness, and a greater use of asthma medications (3–6). All components of the airway wall (adventitia, submucosa, and smooth muscle) have been shown to increase by 10–300% in patients with asthma compared with control subjects (34, 36). Although moderate amounts of airway wall thickening have little effect on the baseline resistance to airflow, it can profoundly affect airway narrowing caused by smooth muscle shortening, especially in peripheral airways (37). Increased airway smooth muscle mass, edema, inflammatory cell infiltration, glandular hypertrophy, and ECM deposition have been shown to contribute to airway wall thickening in asthma (34). With respect to the ECM, and specifically collagen deposition, most studies have focused on changes in the basement membrane (lamina reticularis) because of the availability of biopsy tissues. In contrast, there are limited data on the lamina propria of subjects with asthma, which forms a larger volume of the airway wall, and therefore has the potential to exert a greater effect on airway narrowing (36). Previous studies on the lamina propria of subjects with moderate to severe asthma have demonstrated more collagen antibody staining (11, 38) compared with healthy

control subjects and subjects with mild asthma, whereas Godfrey and colleagues showed no difference (39). One limitation with these studies is that the tissue used was obtained via bronchial biopsies, which makes it difficult to sample the lamina propria consistently between subjects.

In this study, using whole lung tissue and high-resolution NLOM combined with texture analysis, we demonstrate for the first time that the lamina propria in large and small asthmatic airways contains elevated levels of disorganized and fragmented fibrillar collagen. With regard to the structural properties of collagen fibers, it has been shown that the organized assembly of collagen fibrils is essential for the molecule's tensile properties (17). No studies to date have focused on the relationship of collagen organization and the tensile properties of airway tissue; however, the total amount of collagen fibers has been shown to correlate with the resistance and elasticity of parenchymal tissue (40). Furthermore, from surgical models of skin repair it is known that the tensile strength of the repairing tissue increases as collagen organization increases (41). We found no difference in the levels or organization of elastin fibers in asthmatic compared with control airways, which is in line with previous histologic and electron microscopy studies of the lamina propria (11, 39). However, Carroll and colleagues (42) demonstrated by histology that elastin fibers are disorganized in longitudinal bundles associated with mucosal folds.

Changes in individual ECM components or networks as in the case of collagen and elastin have been shown to contribute to changes in airway mechanics through alterations in tissue biomechanics and geometric effects (36, 37). The incorporation of morphologic and physiologic measurements on asthmatic and nonasthmatic tissues into mathematical models has demonstrated that the magnitude of airway wall thickening is sufficient to contribute substantially to asthmatic airway hyperresponsiveness (43). Furthermore, the "perturbed equilibrium hypothesis" suggests that airway wall thickening can destabilize the dynamic forces that control airway caliber, causing airway collapse (44). Despite this, pulmonary measurements in subjects with asthma have shown the

airways to be less distensible (4). Thus, future studies focused on modeling disorganized fibrillar collagen in the asthmatic airway are required to understand truly how this would affect airway closure and airway hyperresponsiveness.

Fibroblasts are the primary cell type responsible for the synthesis and assembly of collagen fibers (45). Although other F-actin-positive mesenchymal cells can produce collagen within the lung, in this study we focused on airway fibroblasts because of their location within the lamina propria adjacent to disorganized collagen, and lack of collagen disorganization located within smooth muscle bundles. The three-dimensional floating collagen I-gel model used in the present study enabled us to mimic the tissue microenvironment, enabling fibroblasts to migrate through the matrix to interact and assemble hydrolyzed collagen I through dendritic extensions, and subsequently via actin stress fibers to induce contraction (30).

Using this model, we demonstrate that compared with control subjects, airway fibroblasts from patients with asthma, but not parenchymal fibroblasts, are abnormal in their ability to form fibrillar collagen I. Our data show that asthmatic airway fibroblasts do not have alterations in cytoskeletal proteins, cell stiffness, or production of collagen I, but do have reduced expression of decorin, a proteoglycan of the small leucine-rich proteoglycan family that binds collagen at β_1 -integrin binding sites and is required for normal spacing of collagen fibrils (46). Furthermore, we also demonstrate *ex vivo* that collagen fibers within the airways of donors with asthma have disorganized collagen fibrils indicative of defective spacing by decorin. In support of our findings, decreased decorin expression has previously been reported within the airways of subjects with asthma (47) and emphysematous tissues in chronic obstructive pulmonary disease (48). Decorin expression has also been shown to be reduced in keloid tissue and hypertrophic burn scars, in which collagen fibrils are known to be less organized compared with normally healed skin (49). Furthermore, decorin knockout mice exhibit abnormal collagen morphology in the skin and tendons because of fibril disorganization (46). Thus, these data support the notion that a reduction in

decorin could contribute to disorganized and fragmented collagen in asthmatic airways, and thus future studies are required to understand the mechanism involved.

In addition to its structural role, collagen topography (synthesis, degradation, organization) is a major effector of the cell microenvironment modifying cell proliferation, adhesion, migration, differentiation, and death (50). The organization of collagen fibers has been shown to profoundly influence the directed migration of leukocytes and cancer cells (51) as well as the migration, senescence, gene expression, and differentiation of dermal fibroblasts (52). Furthermore, it is well understood within the skin literature that collagen fragments promote *de novo* collagen synthesis (53). Thus, defective collagen packaging by airway fibroblasts in asthmatic airways may explain a mechanism whereby fibroblasts residing within an ECM of fragmented collagen could be induced to produce more collagen leading to increased deposition of disorganized collagen and airway remodeling; all without the need for an immune response to promote fibroblast activation and repair following immune cell migration and matrix degradation. Thus, collagen disorganization does not only simply imply thickening of the airway wall, but also may have significant influence on inflammatory and structural cells within the airway, and deserves future investigation.

This study has limitations that should be mentioned. This study used nontransplantable donor lungs from pediatrics and adults with asthma who died of a fatal asthma episode or other unrelated causes. Although such tissue enabled the extraction of cells and inflation of the tissue for analysis, we are unable to characterize the patients with asthma by severity because of only limited information being available on medication use, asthma diagnosis, and viral infections or observe the progression of airway remodeling over time. However, such tissue enabled examination of multiple large and small airways in cross-section, which is essential when investigating the structural organization of ECM molecules. Furthermore, such texture analysis cannot be performed on biopsy tissue taken at the carina because the collagen fibers branch in both directions of the daughter airways, and biopsy forceps deform the tissue structure,

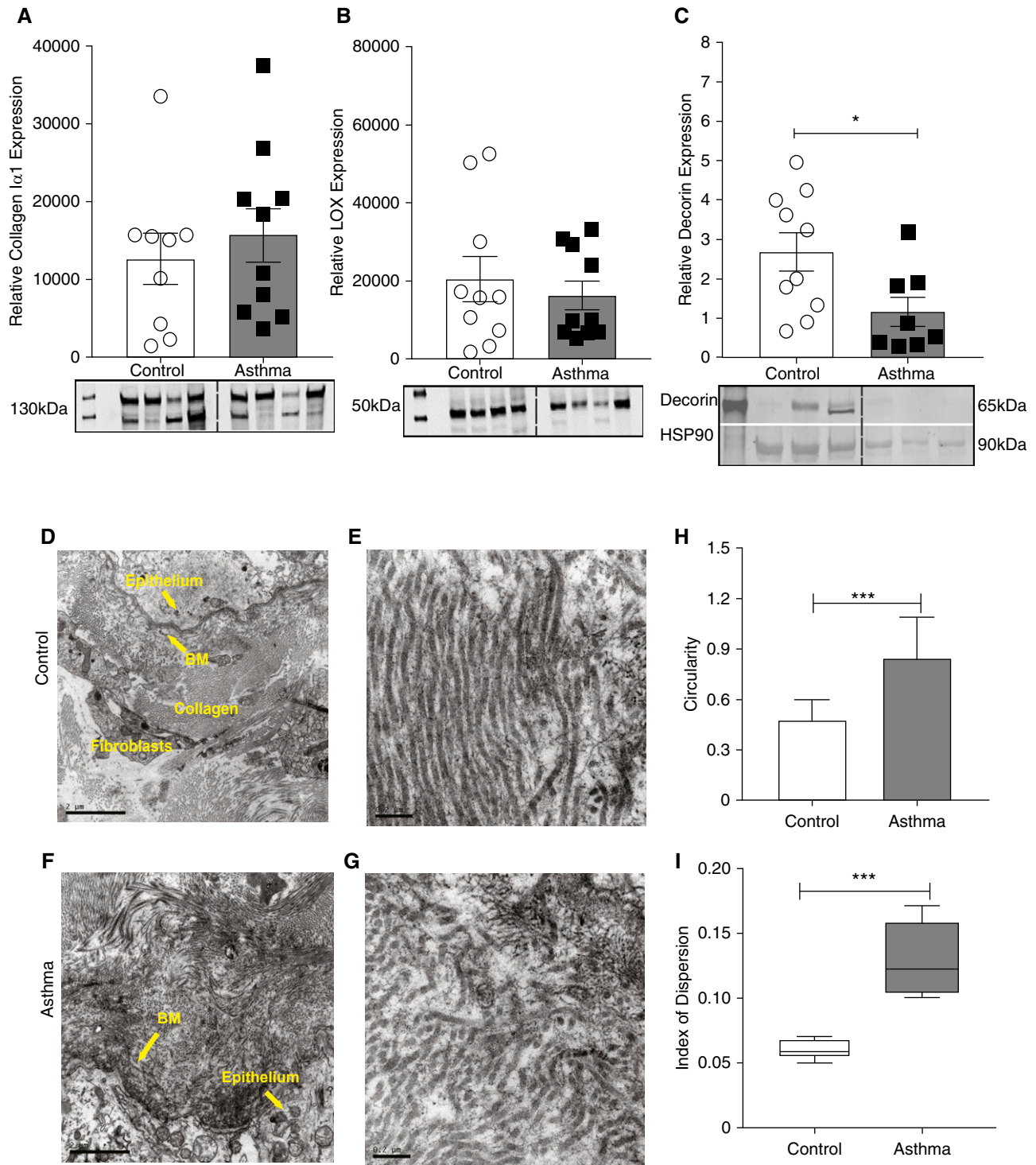


Figure 6. Asthmatic-derived airway fibroblasts are defective at packaging fibrillar collagen I. (A–C) Primary airway fibroblasts from control donors (white bars) and donors with asthma (gray bars) were assessed for protein expression of collagen Iα1 (A), lysyl oxidase (B), and decorin (C). (D–G) Representative low-resolution (D and F) and high-resolution (E and G) transmission electron microscopy images from control (D and E) and asthmatic (F and G) large airways in cross-section. (H and I) Circularity score (H) and dispersion index (I) of collagen fibrils within collagen fibers situated in the lamina propria of control donors and donors with asthma. Scale bars: D and F, 2 μm; E and G, 0.2 μm. Bar graphs represent the means, and error bars denote SEM. **P* < 0.05 and ****P* < 0.001. BM = basement membrane; LOX = lysyl oxidase.

thus making it impossible to perform standardized texture analysis on such tissue samples. Lastly, NLOM enables visualization of fibrillar collagens but does not have the capacity to distinguish between fibrillar collagen types. Because fibrillar collagen types I and III have been shown to be the major fibrillar collagens in both wounded and unwounded lung tissue, with 90% being collagen I (54), we can only infer that the fibrillar collagen signal detected is composed primarily of collagen I.

In conclusion, these findings suggest that the development of airway remodeling in asthma involves disorganization and

accumulation of fibrillar collagen, irrespective of age, sex, or fatal disease. Additionally, these data indicate that defective decorin production by airway fibroblasts could be responsible for the disorganization of collagen fibrils and fibers in the lamina propria. This study expands the understanding of the mechanism of airway remodeling in asthma and highlights urgently needed opportunities for therapeutic intervention. Importantly, several clinical studies have now demonstrated the application of NLOM for use with endoscopy techniques to assess ECM remodeling in cancers and lung tissue

(55, 56). This opens the opportunity for future use of NLOM and texture analysis to assess and monitor airway remodeling in the clinical setting, in response to targeted therapeutic treatments. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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Online supplement

Defective fibrillar collagen organization by fibroblasts contributes to airway remodeling in Asthma

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Supplemental Methods:

Sampling of Lung tissue. A single bronchus and two subsequent airway generations were clamped and dissected from the lung for extraction of airway fibroblasts. Secondly, using a surgical clamp a region of lung parenchyma from the lower lobe was removed for isolation of lung fibroblasts. For full cell culture methods please see below. From the airway and parenchymal tissue, samples were taken for transmission electron microscopy (TEM) analysis, see full methods below. The donor lungs were then fully inflated with cryomatrix and frozen using liquid nitrogen vapor. While still frozen, each explanted lung was cut into contiguous 2 cm thick slices in the trans-axial plane, generating 8 – 10 slices depending on the size of the lung, as previously described(1, 2). A uniform random sampling method was applied to select sampling sites using a line grid (225mm²) that was superimposed onto the photographs of each lung slice. Sample locations (line intersection) falling on connective tissue or large airways and blood vessels were also excluded from the sampling. Three 15mm x 20 mm cylindrical tissue cores were taken for formalin fixation and paraffin embedding (FFPE). FFPE samples were sectioned using a microtomb at 5 µm thickness and mounted on SuperFrost slides (12-550-123, Thermofisher). Slides were then de-paraffinized using 2 changes of xylene (X5SK-4, FisherScientific) for 5 minutes each and rehydration through graded levels of ethanol (100%, 95% and 70% in distilled water) for 3 minutes each. The first sequential section from each tissue block was stained with Movats pentachrome stain and then digitally scanned (0.25 microns per pixel X40 scans) using a ScanScope XT slide scanner (Aperio Technologies). All airways were identified and segregated into small (< 2mm diameter) or large (> 2 mm diameter) airways. There was no statistical difference in the mean small airway diameter for non-asthmatics (704.1 µm ±83.1) versus asthmatics (753.1 µm ±117.2), or for the mean size of large airways in non-asthmatics (5.81 ± 0.98

mm) and asthmatics (5.3 ± 1.27 mm). The second sequential section was stained with hematoxylin (CA1.05174.0500, Sigma) and used for non-linear optical microscopy imaging.

Non-linear optical microscopy (NLOM) imaging of tissue. Lung tissue samples were imaged using an *in-house* built multimodal femtosecond non-linear optical microscope (NLOM)(3, 4), capable of acquiring co-localized two-photon excited fluorescence (TPEF) and second harmonic generation (SHG) images simultaneously. SHG signal was collected in the forward-direction while TPEF was collected in the backscattered (epi-direction) (3, 4). The light source for TPEF and SHG imaging was a Ti:Sapphire oscillator laser (Tsunami, Spectra-Physics) operating at 800 nm with a 100 fs pulse duration and 20 nm bandwidth. Average output power was measured to be 1W when the oscillator was pumped with a 7.25W of green light at 532nm (Millennium Pro, Spectra-Physics). A Faraday isolator (Newport) was placed close to the emission exit of the Ti:Sapphire oscillator to prevent any back-reflections of the femtosecond pulses from re-entering the laser cavity. Re-compression of the pulses was accomplished using a pair of chirped laser mirrors (Layertec GmbH, Germany) to compensate for the large positive group velocity dispersion (GVD) introduced by the Faraday isolator and other microscope optics. The collected NLO signals were transmitted to two non-descanned PMT detectors (H9656 series, Hamamatsu, Bridgewater, NJ, USA) mounted on the microscope assembly for simultaneous TPEF and SHG signals through arrays of dichroic and colour filters (A10034 series, Hamamatsu Photonics, Bridgewater, NJ, USA) at 530 ± 20 nm and 400 ± 30 nm, respectively. Typically, 25mW of pump (measured after the objective) was used for imaging. Two objective lenses were used for tissue imaging: 20x, 0.75NA infinity corrected air objective lens (Olympus Canada, Markham, ON, Canada) and a 10x, 0.40NA infinity corrected air objective lens (Olympus Canada, Markham, ON, Canada). The

images used for the texture analysis presented in this work were acquired with the 20x, 0.75NA infinity corrected air objective lens, which gives a maximum scan size of 450x450 μ m (or 512 x512 pixels). The pixel dwell time was in average 21 μ s per pixel, and 8 frames were acquired and averaged to improve the signal-to-noise ratio. For each donor, 4 airways in total were imaged, and individual SHG and TPEF images were acquired simultaneously from three randomly selected regions of interest (ROIs).

Image processing & collagen fiber texture analysis. Post image processing was performed in ImageJ software. Image background correction, intensity normalization and calculation of various image texture parameters were carried out using MATLAB 7.5. First order statistics (FOS) textural parameters, and circularity were calculated using Fiji's analysis toolbox and plugins(5, 6). A custom-built texture analysis toolkit based on some of the texture analysis functions available in the MATLAB image processing toolbox was used to calculate second order statistics parameters using a gray level co-occurrence matrix (GLCM) parameters(7, 8), entropy (a measure of the degree of disorder of the structures within an image) and inverse difference moment (IDM, a metric related to the homogeneity of the structures within the image) (9). The co-occurrence matrix was calculated in four orientations: horizontal, vertical, and the two diagonals (directions defined by four angles: 0, 45, 90, and 135 degrees); to avoid bias regarding the sample orientation when placing them on the microscope stage for imaging, the average value considering all directions was calculated. A window size with 8 pixels including all direct neighbours of each evaluated pixel was adopted to extract features from 8 bit images. Data from the 3 ROI's per airway were averaged, and then the 4 airways averaged per subject.

Fiber directionality analysis. Fibers were identified using a method for unbiased detection of curvilinear structures based on ridge detection(10). By locating the first and second directional derivatives of the image for the extraction of the line points, it was possible to further calculate fiber thickness, over-lap and spacing. Fiber directionality analysis was used to quantify the preferred orientation of fibrillar collagen and elastin fibers within the airway tissue. The method used is based on the work of Liu et al(5)[ref], which introduced a new method for linear pattern extraction and directional analysis based on Fourier spectrum analysis. SHG and TPEF images (collagen and elastin, respectively) from three randomly defined ROIs in the lamina propria region of small and large airways were used. For each image, collagen and elastin fibers with a preferred orientation generate a periodic pattern in the Fourier transform of the image. The images were then sub sectioned into square pieces, and their Fourier power spectra is computed. The Fourier spectra are analyzed in polar coordinates, and the power is measured for each angle using Sobel filter. The gradient of each image is calculated using a 5x5 Sobel filter, and is used to derive the local gradient orientation. This orientation is then used to build the histogram. A Gaussian curve is fitted on the histogram and the standard deviation is associated with the angular dispersion of the structures analyzed. Data from the 3 ROI's per airway were averaged, and then the 4 airways average per subject.

Primary fibroblast culture. Airway and parenchymal lung tissue was obtained from each donor lung as described above in the lung sampling section. Primary airway and lung fibroblasts were then obtained using the outgrowth technique as previously described(11, 12). Briefly, bronchi and pleura-free lung parenchyma were rinsed in cold PBS without Ca^{2+} and Mg^{2+} (PBS, 14190250, Gibco) to remove mucus plugs and all traces of blood. Airway and lung parenchymal tissue were

then diced into 1mm³ pieces and placed in 6 well tissue culture plates (10062-892, VWR) with 1 ml Dulbecco's Modified Eagle's medium containing 2mM L-glutamine (DMEM, 11965-118, Gibco), supplemented with 10% fetal bovine serum (FBS, 12483012, Gibco) and 1% antibiotic/antimycotic solution (SV3007901, Hyclone). The explants were then kept at 37°C in a 5% humidified CO₂ atmosphere, and the growth medium was replenished every 3 days to rid the culture of non-adherent cells and tissue debris. Outgrowth of fibroblasts from the tissue explants occurred within 7 days, after which tissue explants were carefully removed using a p20 pipette tip. Primary airway and lung fibroblasts were then harvested using trypsin/EDTA (CC-5012, Lonza) and used at passage 1 -2 for the experiments described below.

Collagen I gel contraction assay. Free floating 3-dimensional collagen gels confer a mechanically compliant environment which enables fibroblasts to compact collagen matrix and transmit mechanical force into the surrounding matrix inducing local and global matrix remodeling(13). This collagen gel contraction assay is used as a functional measure of fibroblast repair(13). Collagen gels were made as previously described in triplicate for each donor(14). Briefly, 12 well tissue-culture plates were coated with 1% bovine serum albumin (BSA, A9418-10G, Sigma) in DMEM (Gibco) for 2 hours. The medium was removed and 1 ml of 0.4 mg/ml type I rat tail collagen I (354236, Corning) in DMEM (Gibco) was added and allowed to polymerize for 16 h at 37°C. Collagen I gels were then detached from the sides of the plate using a p2 pipette tip before primary airway fibroblasts were seeded on top of the gel at a density of 40,000 cells in the presence or absence of 10 ng/ml transforming growth factor (TGF)- β . Fibroblast contraction of collagen I gels was then followed over 72 hours with light microscopy and quantified to measure the change in gel area using Image J software. One of the triplicate gels was then fixed in 4%

paraformaldehyde for 20 minutes and used for imaging and one was harvested with protein extraction buffer (PEB, FNN0011, ThermoFisher) for protein analysis (see detailed methods below).

Immunofluorescent staining of collagen I gels and formalin fixation and paraffin embedded-tissue sections. To visualize fibroblasts in fixed collagen I gels and FFPE sections, samples were incubated with Phalloidin (A12379, ThermoFisher) in PBS (10010015, Gibco) for 1 hour 30 minutes to stain F-actin to visualize the cytoskeleton and with nuclear stains, 4',6-diamidino-2-phenylindole (DAPI, D8417, Sigma) in PBS (10010015, Gibco) for cells in gels and propidium iodide (P4170, Sigma) for cells in FFPE sections. Collagen I gels were then imaged in TBS using Nunc™ glass bottom dishes (150682, ThermoScientific). Tissue sections were washed three times in tris buffered saline (TBS, T5912, Sigma) for 10 minutes each. FFPE slides were dehydrated through graded levels of ethanol (70%, 95% and 100% in distilled water) for 3 minutes each and allowed to air dry. Tissue sections were then mounted in Cytoseal™ (23-244-256, ThermoFisher). Collagen gels were also imaged using the same *in-house* built multimodal femtosecond non-linear optical microscope, and the same parameters described in the previous section. The images used for all the analysis related to collagen gels presented in this work were acquired with the 20x, 0.75NA infinity corrected air objective lens (Olympus Canada, Markham, ON, Canada), which gives a maximum scan size of 450x450µm (or 512 x512 pixels). Individual SHG and TPEF images were acquired simultaneously from each gel, from five randomly selected regions of interest (ROIs).

The number of fibroblasts present in each collagen gel was assessed by counting nuclei stained with DAPI ((4',6-diamidino-2-phenylindole) using a Fiji customized macro (Bolte et al).

In summary, a threshold was adjusted in order to highlight the nuclei, and then allow the binarization of all images. A watershed filter was applied, and a 1 pixel line was added on the cases when some cells were positioned too close together. The images were then filtered again to ignore structures with less than a certain number of pixels (we used 5). The number of nuclei were then identified based on the object counting plugin(15).

Confocal Microscopy. Confocal images and SHG-NLOM images were collected using a custom Zeiss LSM 880 inverted confocal microscope (Carl Zeiss Inc.). Each confocal channel (543 nm laser to excite Propidium Iodide, and 488 laser to excite Phalloidin) and the SHG (Coherent Chameleon Ultra II tunable femtosecond oscillator (Coherent Inc.), set to a wavelength of 800 nm)) for fibrillar collagen were collected sequentially to minimize cross-talk and photo-bleaching. Scattered light was rejected using a 488/543 notch filter (Carl Zeiss MBS 488/543) and a 405 notch filter (Carl Zeiss MSB 405), respectively. Fluorescence was collected in the backward direction through a 1 AU pinhole using a 880's filter-free QUASAR detection system (Carl Zeiss Inc.) that allows the detection windows for each fluorophore to be set dynamically. SHG was measured using the internal QUASAR detector in the backward direction with the detection range set to 390nm-410nm, using a shortpass filter (Carl Zeiss VS-MP 690+) to reject the scattered laser light. SHG measurements were taken with the pinhole set to maximum (600 μm), and a detector gain of 750. All images were collected using a 20x/NA 0.8 plan achromat lens (Carl Zeiss, Plan-Apochromat 20X/0.8 Wd = 0.55 M27), with a 450x450 μm field of view at a pixel density of 512x512.

Immunoblotting. Primary airway fibroblast cell lysates were collected from cell-seeded collagen gels and collagen I coated plates by the use of protein extraction buffer (PEB), (FNN0011, Thermofisher) containing protease (P2714-1BTL, Sigma) and phosphatase inhibitor cocktails (P5726-1ML, Sigma) as well as phenylmethanesulfonyl fluoride (PMSF, P7626, Sigma) as described previously(12). The harvested cell lysates were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) by loading onto SDS-polyacrylamide gels (4561096, Mini-PROTEAN TGX) with standard molecular weight ladder as a control. Separated proteins were then blotted on a nitrocellulose membrane. After protein transfer, specific proteins of interest were identified with validated antibodies. Protein expression of collagen I α 1 (ab34710, Abcam), non-muscle myosin IIB (ab684, Abcam), β -tubulin (05-661 (CH), Millipore) lysyl oxidase (ab174316, Abcam), lysyl oxidase like 1 (PA5-79609, Thermofisher) and lysyl oxidase like 2 (ab96233, Abcam) were determined and normalized to total protein by the use of the REVERT normalization protocol (926-11015, Licor) while the expression of decorin (ab67449, Abcam) was normalized to the housekeeping protein heat shock protein 90 (HSP90, BD610418, BD Biosciences). Data are presented as relative protein expression.

mRNA analysis. Primary airway fibroblasts were collected from on collagen I-coated plates with RLT buffer (79216, Qiagen) and RNA extraction done with the RNeasy Mini Kit (74104, Qiagen). cDNA synthesis was then performed with the High Capacity cDNA Reverse Transcription Kit (4368814, Applied Biosystems) according to the manufacturer's instructions. Real-Time quantitative polymerase chain reaction (qRT-PCR) was carried out with the ViiA 7 Real-time PCR system. This was done with the TaqMan universal PCR master mix (4304437, Thermofisher) and already-made Taqman® Gene expression assays in 384 well-plates as per the instructions of the

manufacturer. Expression levels of specific genes of interest lysyl oxidase (Hs00942480_m1, ThermoFisher), Decorin (Hs00754870_s1, ThermoFisher), Collagen I α 1 (Hs00264051_m1, ThermoFisher) and glioma-associated oncogene (GLI-1, Hs00171790_m1, Thermofisher) were normalized to the expression of housekeeping genes; protein phosphatase 1 catalytic subunit, alpha isoenzyme (PP1 α , Hs00267568_m1, Thermofisher), GAPDH (Hs02786624_g1, Thermofisher) and β 2-microglobulin (Hs00984230_m1, Thermofisher). The normalized relative gene expression values was calculated and presented at $2^{-\Delta Ct}$.

Optical magnetic twisting cytometry (OMTC). Fibroblast stiffness was measured using OMTC as previously described(16, 17). Briefly, cells were seeded at passage 2 and grown to confluency on a 96 well plate with surface bound magnetic beads. Cells were then serum deprived and treated with control media or recombinant transforming growth factor (TGF)- β 1 for 24 hours. The 96 well plate was then placed on the stage of an inverted microscope (DM-IRB, Leica Microsystems) equipped with an electro-magnetic twisting device and a charge-coupled device camera (1,280 $\times$ 1,024 pixels, 12-bit gray scale, SensiCam, The Cooke, Auburn Hills, MI). Beads were twisted with a specific torque of 56 Pa at 0.5 Hz, and the camera imaged the beads continuously (~200 beads at a time) at 16 frames per twisting cycle. Using an intensity centroid algorithm, the bead positions in each recorded image were automatically determined(16) and Fourier transformation was used to extract the displacement of each bead in response to the applied torque(16-18). Beads with erratic, irreproducible motions were not analyzed. Thus, for a given specific torque ($\tilde{T}$) applied to a bead and the resultant bead displacement ($\tilde{D}$), as described above, a complex stiffness ($\tilde{G}$) of the cell was defined as the ratio of the torque to the displacement, i.e., $\tilde{G} = (\tilde{T}/\tilde{D}) = G' + iG''$, where

G' is the in-phase component or elastic stiffness, which we hereafter refer to as stiffness (in Pascal/nm).

Transmission electron microscopy (TEM) imaging. Samples were fixed in 2.5% glutaraldehyde (18401, Ted Pella, USA) for 2 hours and then washed with 0.1M cacodylate buffer (18851, Ted Pella, USA). Next, the samples were transferred to a solution of 1% osmium tetroxide (Cat#18456, Ted Pella, USA) and 1% potassium ferrocyanide (P236-500, Fisher, USA) for 1 hour, and then washed with distilled water. The samples were dehydrated through a graded acetone series (A18P-4, Fisher, USA) and embedded in epoxy resin (18010, Ted Pella, USA). The resin blocks were sectioned with a diamond knife on a Leica EM UC7 ultramicrotome (Leica Microsystems, Canada). Sections were picked up on Formvar (19223, Ted Pella, USA) coated grids and stained with lead citrate (19314, Ted Pella, USA,) and uranyl acetate (19481, Ted Pella, USA)(19). TEM images of airway tissue sections were obtained with an electron microscope (Philips/FEI Tecnai 12 TEM) equipped with a digital camera (Gatan 792) at a magnification of 37,000X. Capturing a whole cross-sectional image of a collagen fiber often required multiple images that were merged together. A band-pass filter was applied to the TEM images to reduce the background noise. Each individual collagen fibril or “particle” within the collagen fiber was assigned a number from 0 to 1, (0 being a perfect line and 1 a perfect circle). The index of circularity for each particle was calculated using a MATLAB routine.

Supplemental Figure legends

Figure E1. NLOM demonstrates fibrillar collagen is not disorganized in smooth muscle region of airways. (A) Representative low and high magnification regions of interest of the SHG and TPEF signal from the smooth muscle region of control and asthmatic airways. Graphical analysis of (B-D) collagen (SHG signal) and (E-G) elastin (TPEF signal) textural analysis features intensity, entropy and inverse difference moment (IDM) imaged in the smooth muscle of large airways from asthmatic and control donors. Bar graphs represent the mean and error bars denote S.E.M. Circles and squares depict data from 12 biologically independent control donors (total of 48 airways, average of 4/donor, 3 ROIs/airway), 13 biologically independent asthmatic donors (total of 52 airways, average of 4/donor, 3 ROIs/airway). $**P < 0.01$.

Figure E2. Structural characteristic of elastin fibers in remodeled airways. Representative regions of interest (ROI) from NLOM-Two photon excitation fluorescence (TPEF) images of the lamina propria of small and large airways showing elastin fibers and its colour-coded orientation from (A, B) control and (E, F) asthmatic donors. Representative histograms show the preferred direction of elastin fibers in small airways from (C) control and (G) asthmatics donors, as well as for large airways from (D) control and (H) asthmatic donors. (I) Graphical analysis of the dispersion angle for controls (white bars) and asthmatics (grey bars) in small and large airways. Bar graph represent the mean and error bars denote S.E.M. Circles and squares depict data from 12 biologically independent control donors (total of 48 airways, average of 4/donor, 3 ROIs/airway), 13 biologically independent asthmatic donors (total of 52 airways, average of 4/donor, 3 ROIs/airway).

Figure E3. Asthmatic airway fibroblasts are not defective in response to TGF β 1 signaling.

(A) Primary airway derived from control and asthmatic donors were seeded in rat-tail collagen I gels and allowed to contract for 72 and 144 hours in the presence or absence of 10ng/ml TGF- β 1 (n=6 per group), and contraction was assessed as percent gel reduction. (B) Primary airway fibroblasts from control and asthmatic donors were assessed with and without 10ng/ml transforming growth factor (TGF) β 1 for cell stiffness (G1 (Pa/nm) using optical magnetic twisting (n=6per group). (B) Bar graphs represent the means and error bars denote S.E.M.

Figure E4. The protein expression of the collagen cross-linking enzymes lysyl oxidase like 1 and lysyl oxidase like 2 in airway fibroblasts is unaltered. Primary airway fibroblasts from control (white bars) and asthmatic (grey bars) donors were assessed for protein expression of (A) lysyl oxidase like 1 (LOXL1) and (B) lysyl oxidase like 2 (LOXL2) and normalized to total protein.

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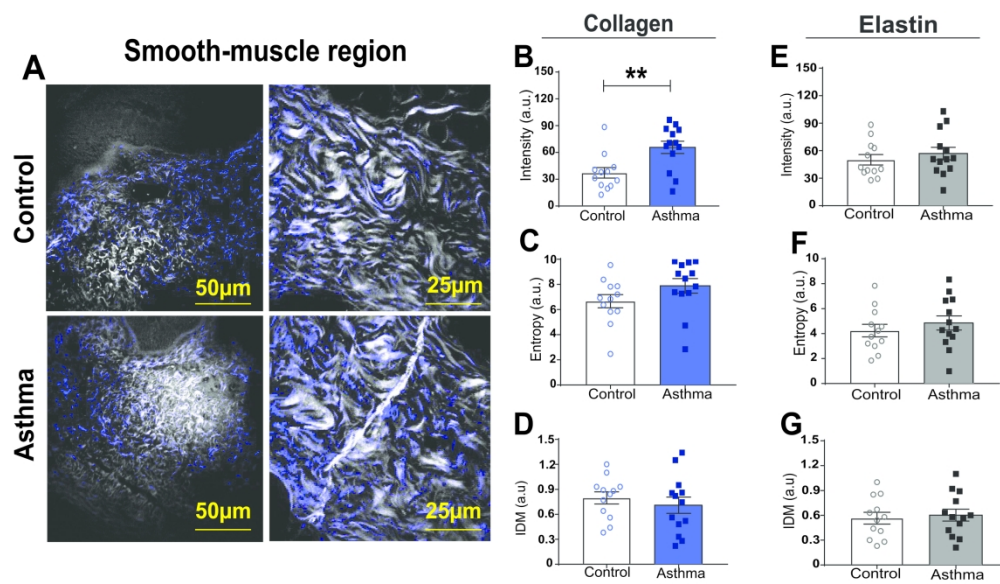


Figure E1

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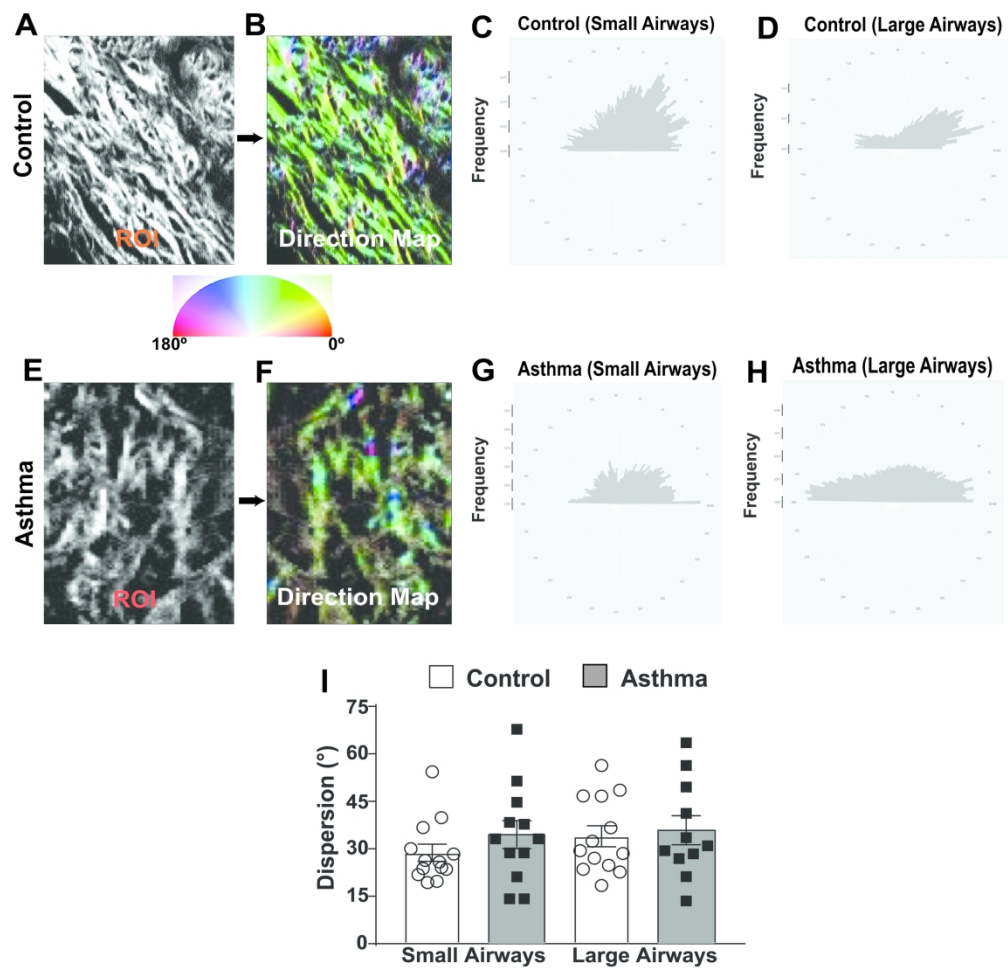


Figure E2

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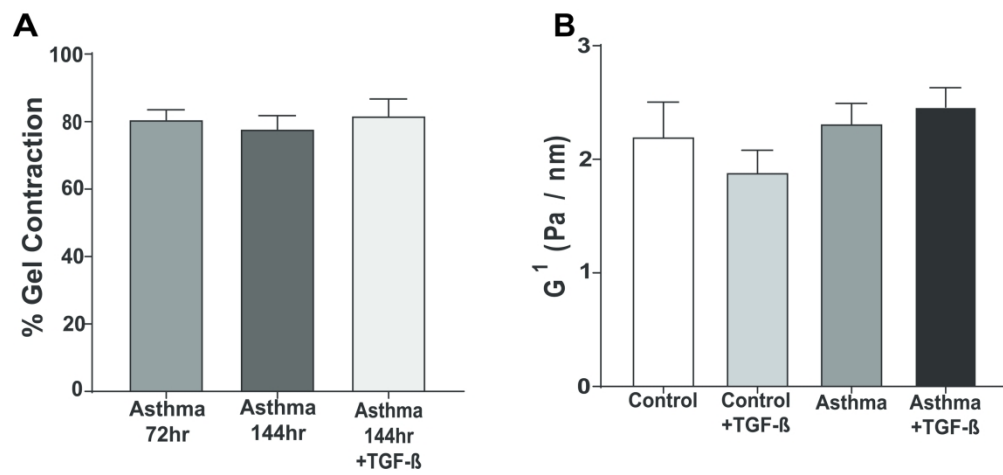


Figure E3

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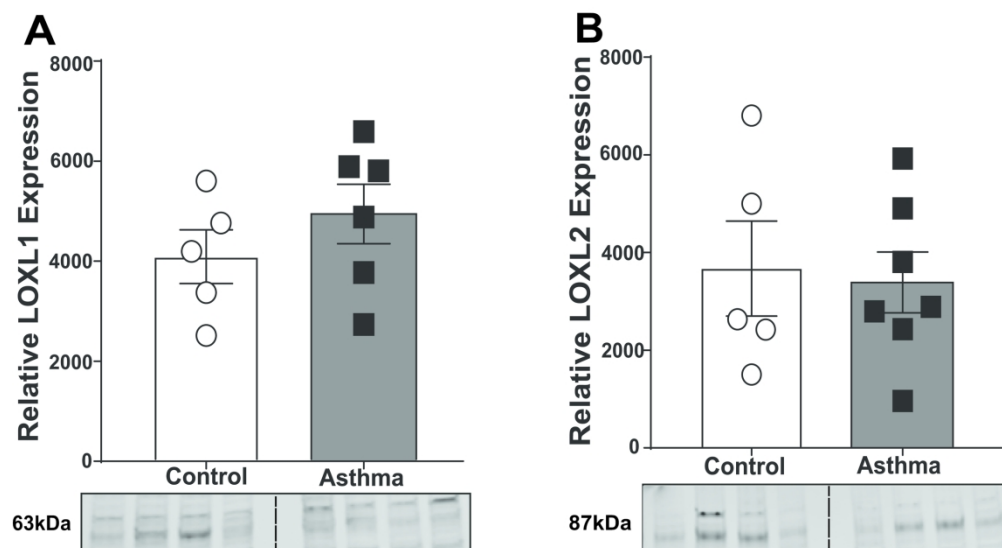


Figure E4

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