

Reduction of Tumstatin in Asthmatic Airways Contributes to Angiogenesis, Inflammation, and Hyperresponsiveness

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Rationale: Angiogenesis is a prominent feature of remodeling in asthma. Many proangiogenic factors are up-regulated in asthma, but little is known about levels of endogenous antiangiogenic agents. Collagen IV is decreased in the airway basement membrane in asthma. It has six α chains, of which the noncollagenous domain-1 domains have endogenous antiangiogenic properties.

Objectives: To study the expression of the noncollagenous domain-1 of the $\alpha 3$ chain of collagen IV, tumstatin, in the airways of subjects with and without asthma and to examine the potential for tumstatin to regulate angiogenesis and inflammation.

Methods: We used immunohistochemistry and dot blots to examine the expression of tumstatin in bronchial biopsies, bronchoalveolar lavage fluid, and serum. We then used an *in vitro* angiogenesis assay and a murine model of allergic airways disease to explore tumstatin's biological function.

Measurements and Main Results: The level of tumstatin is decreased 18-fold in the airways of patients with asthma but not in subjects without asthma, including those with chronic obstructive pulmonary disease, cystic fibrosis, and bronchiectasis. *In vitro*, recombinant tumstatin inhibited primary pulmonary endothelial cell tube formation. In a mouse model of chronic allergic airways disease, tumstatin suppressed angiogenesis, airway hyperresponsiveness, inflammatory cell infiltration, and mucus secretion and decreased levels of vascular endothelial growth factor and IL-13.

Conclusions: The observation that tumstatin is decreased in asthmatic airways and inhibits airway hyperresponsiveness and angiogenesis demonstrates the potential use of antiangiogenic agents such as tumstatin as a therapeutic intervention in diseases that are characterized by aberrant angiogenesis and tissue remodeling, such as asthma.

Keywords: asthma; angiogenesis; collagen; type IV collagen $\alpha 3$ chain

Asthma is an inflammatory disease characterized by airway remodeling. A prominent feature of this remodeling is angiogenesis, which is an increase in the number and size of the blood

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Asthma is an inflammatory disease characterized by airway remodeling. A prominent feature of this remodeling is angiogenesis, which is an increase in the number and size of the blood vessels. The underlying mechanisms of angiogenesis remain unknown.

What This Study Adds to the Field

The molecule tumstatin is absent from the airways of patients with asthma. *In vitro*, tumstatin inhibited pulmonary endothelial cell tube formation in a dose-related manner, identifying a potential role in attenuating angiogenesis. In a murine model of chronic ovalbumin-induced allergic airways disease tumstatin inhibited angiogenesis and accumulation of vascular endothelial growth factor in the airway. Moreover, it attenuated the development of airway hyperresponsiveness and the accumulation of inflammatory cells and increases in IL-13 levels in the airways.

vessels (1, 2). Chronic inflammation, such as that observed in asthma, is often accompanied by vascular remodeling and angiogenesis (3). The excessive blood vessel formation and expansion occurs in conjunction with fibrosis of inflamed tissues, leading to aggravation of chronic inflammation. Although angiogenesis occurs in asthma, its underlying mechanism is unknown.

It appears that inflammation and remodeling are interdependent processes that influence the clinical long-term evolution of asthma (4), but it is not known if, in the airways of patients with asthma, remodeling initiates inflammation or *vice versa*. Although airway wall remodeling may occur as the result of uncontrolled inflammation (5), these two events may occur in parallel.

Angiogenesis can be initiated by endogenous angiogenic factors released from mesenchymal cells and/or inflammatory cells. Endogenous angiogenic inhibitors, including tumstatin (the NC1 domain of the $\alpha 3$ chain of collagen IV), are present in fluids and tissues in the body (6). Under physiological conditions, angiogenesis is controlled by an equilibrium between proendogenous and antiendogenous angiogenic factors that can be released from the extracellular matrix and then become bioavailable (7).

Collagen IV is a major component of airway basement membrane. It has six α chains, each consisting of a 7S domain at the amino terminus, a central collagenous domain, and a NC1

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domain at the carboxyl terminus (8). Several of the α -chain NC1 domains of the collagen IV molecule have antiangiogenic properties, including tumstatin, which is antiangiogenic when cleaved from the $\alpha 3$ chain. MMP-9, which is increased in asthmatic airways (9), cleaves the $\alpha 3$ NC1 domain, releasing active tumstatin (10). Tumstatin inhibits angiogenesis via blockade of the interaction of vascular endothelial growth factor (VEGF) with the $\alpha_v\beta_3$ integrin (11), causing apoptosis of proliferating endothelial cells (10).

Up-regulation of many proangiogenic factors (12–14) has been reported, whereas collagen IV is decreased in the airways of patients with asthma (15, 16). Angiogenesis may occur in asthma as a result of raised levels of proangiogenic elements, but there have been no reports about the levels of tumstatin in the airways or whether it functions as an angiogenic inhibitor in the lung. The aim of this study was to investigate the presence of tumstatin in the airways of volunteers with and without asthma and to examine the potential for tumstatin to regulate angiogenesis. In addition, we assessed its effects on angiogenesis and inflammation in a mouse model using IL-13 as a sentinel cytokine (17–21). Some of the results of these studies have been previously reported in the form of abstracts (22, 23).

METHODS

Study Population

The study was approved by the Ethics Review Committee of the South West Sydney Area Health Service, Royal Prince Alfred Hospital and The University of Sydney human research ethics committee. Written informed consent was obtained from all subjects.

Samples were obtained from 25 subjects with asthma and 44 subjects without asthma. Full details are provided in Table E1 in the online supplement.

Collection and Preparation of Samples

Lung tissue (from surgical tailings or explanted lungs) was fixed in formalin and embedded in paraffin. Bronchial biopsies and 50 ml of bronchoalveolar lavage fluid (BALF) were obtained by flexible fiberoptic bronchoscopy. Two or three bronchial biopsies were placed in Hanks' balanced salt solution for immediate dissection of the airway smooth muscle (ASM) bundles for culture. ASM cells were isolated as previously described (24). The remaining biopsy samples were snap frozen in Optimal Temperature Cutting embedding medium (Fronine Laboratory Supplies, Riverstone, Australia) and stored at -80°C . Human pulmonary endothelial cells were isolated from explanted lungs. Briefly, endothelial cells were isolated from blood vessels by enzymatic digestion. Fibroblasts were isolated from explanted lungs and lung resections as previously described (25, 26).

Immunohistochemical Detection of Blood Vessel Area and Number in Human Samples

Paraffin-embedded airway sections were stained for CD31 (PECAM-1) and von Willebrand factor (vWF) (both from Santa Cruz Biotechnology Inc., Santa Cruz, CA) to detect a positive signal for endothelial cells and blood vessels, respectively. Sections were imaged using an Olympus BX51 microscope (Olympus, Tokyo, Japan) and processed using Leica imaging software IM1000 (Leica, Wetzlar, Germany).

Detection of Collagen IV $\alpha 1$ to $\alpha 6$ Noncollagenous Domains

Paraffin-embedded bronchial tissue sections or sections from fresh frozen biopsies from subjects with and without asthma were stained for the six collagen IV α chain NC1 domains (collagen IV $\alpha 1$ – $\alpha 6$ NC1 was a kind gift from Dr. Sado Shigei, Medical Research Institute, Okayama, Japan) (27). Bronchial tissue from patients with COPD, cystic fibrosis, and bronchiectasis were stained for collagen IV $\alpha 3$ NC1. Sections were imaged as described above. In addition, sections from

fresh frozen biopsies from subjects with and without asthma were stained for collagen IV $\alpha 1$, $\alpha 3$, and $\alpha 5$ NC1. The specificity of the antibody was confirmed by preabsorption of recombinant tumstatin using dot blots from BALF derived from subjects with and without asthma (*see* Figure E1 in the online supplement).

Dot Blot

For dot blots performed on biopsy-derived tissue, four biopsies from subjects with asthma and four from subjects without asthma were placed in 1 ml 0.5 M acetic acid (MilliQ water) and sonicated for 5 minutes (60% maximum amplitude, 6s/4s pause). The sample was concentrated with a 10,000-kD filter tube (Millipore, Billerica, MA) for 15 minutes at $4,000 \times g$. Samples were kept on ice until use (20–30 minutes). Protein levels were estimated by measuring absorbance at 280 nm (NanoDrop, Wilmington, DE). Equal amounts of protein were loaded onto the membrane.

Col IV $\alpha 1$ and $\alpha 2$ were used as a loading control.

Colocalization of Tumstatin and VEGF

Paraffin sections were stained for tumstatin (rat antihuman collagen IV $\alpha 3$ NC1; Shigei Medical Research Institute, Okayama, Japan) and VEGF (rabbit antihuman VEGF₁₆₅; Chemicon International, Temecula, CA). Images were acquired using the LSM 510 Meta confocal microscope (Zeiss, Carl Zeiss AC, Oberkochen, Germany).

Endothelial Cell Tube Formation

Endothelial cell tube formation in the presence and absence of recombinant tumstatin was assessed using a commercial tube formation assay (BD BioCoat Angiogenesis system-endothelial cell tube formation; BD Biosciences, San Jose, CA). Images were taken using a Camedia C-4000 Zoom digital compact camera (4.0 megapixel) (Olympus). The number of tubes per well was determined and statistical analysis (one-way analysis of variance [ANOVA] with Dunnett's post test) was performed.

3D Matrigel

Endothelial cells were seeded onto 12-well plates precoated with 400 μl of Matrigel (BD Biosciences) at 4×10^4 cells/ml in 10% FBS/F-12 with endothelial cell growth supplement (BD Biosciences) in the presence of VEGF (100 ng/ml), angiopoietin-1 (100 ng/ml), ephrin-B2 (300 ng/ml) (all from R&D Systems), and 5% fibrin (Sigma-Aldrich, St. Louis, MO). Tumstatin was added at Time 0 and every 48 hours during the 7-day incubation. Images were captured using a digital Olympus Camedia-4000 Zoom Digital Compact Camera (4.0 megapixel) every day for 7 days.

Western Blotting

A nonreducing Western blot was used to detect tumstatin in serum samples. Samples were electrophoresed on a 4 to 20% gradient gel (BioRad, Hercules, CA) and transferred to a polyvinylidene fluoride membrane. Immunodetection of tumstatin was performed, and protein bands were imaged and quantified as for dot blotting.

Inhibition of Proliferation

We used tumstatin active component peptides T3 and T7 (28) at 4.5 μM . T3 was dissolved in water and T7 in 4% acetonitrile (Phoenix Pharmaceuticals, Burlingame, CA). The peptides or appropriate controls were added to ASM cells from subjects with and without asthma, and fibroblasts and primary pulmonary endothelial cells from subjects without asthma were added. The peptides were replaced after 24 hours and then after every 48 hours. Proliferation was assessed using an MTT assay (Sigma-Aldrich) at Days 3, 7, and 9.

Full details of all protocols are provided in the online supplement.

Mouse Model of Chronic Ovalbumin-induced Allergic Airways Disease

A chronic model of allergic airways disease (AAD) was generated as described previously (29, 30). Some groups of mice were adminis-

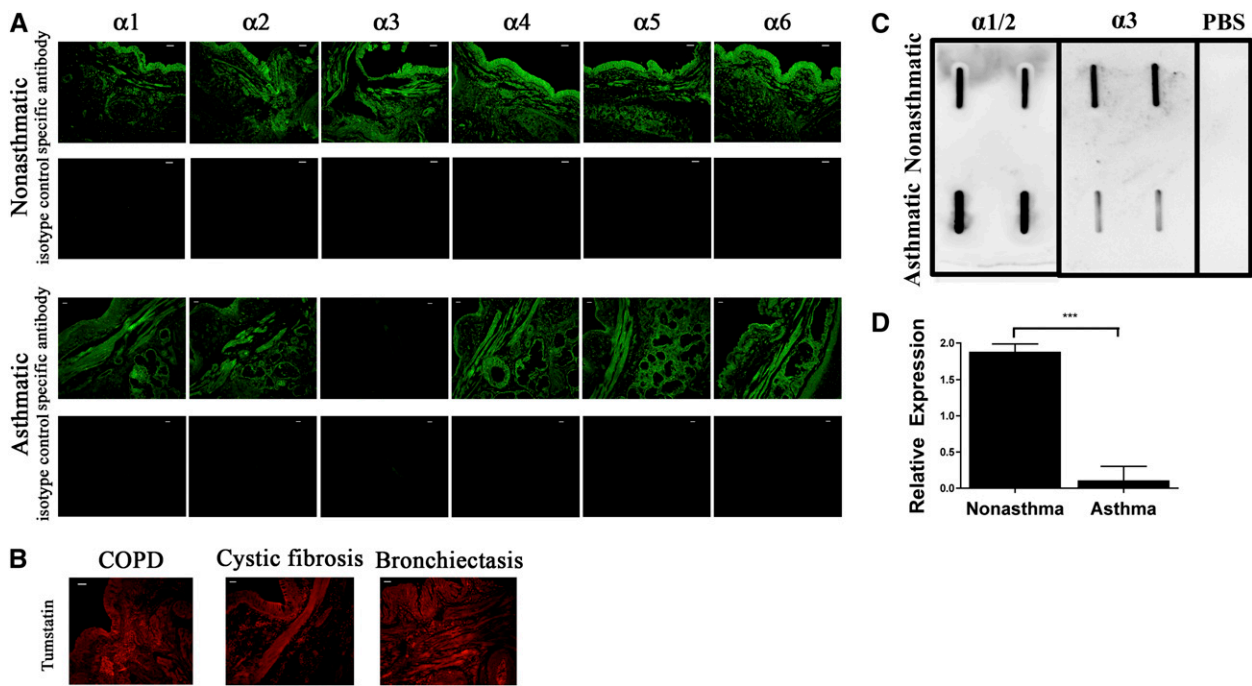


Figure 1. Immunofluorescent detection of collagen IV α chain noncollagenous domain-1 (NC1). (A) Paraffin-embedded airway sections from subjects with and without asthma were stained with specific antibodies to the collagen IV α chain NC1 domains or an IgG₁ isotype control. Green fluorescence represents tumstatin-specific staining. Images are representative of results obtained from four subjects without asthma and five subjects with asthma. (B) Detection of tumstatin in paraffin-embedded airway sections from individuals with COPD, cystic fibrosis, and bronchiectasis. Red fluorescence represents specific staining. Images are representative of results obtained from eight patients with COPD, four patients with cystic fibrosis, and one patient with bronchiectasis. In all images, the scale bar equals 10 μ m. (C) Tissue was pooled from four asthma-derived and four nonasthma-derived biopsies. Collagen extracted and dot blots were performed for tumstatin and for total collagens ($\alpha 1$ and $\alpha 2$). (D) Mean results for n = 3 separate blots performed in duplicate. ****P* < 0.001.

tered tumstatin IN at 300 or 600 ng in PBS per 20 g body weight or PBS alone once a day from Day 25 to Day 115. The airway resistance to inhaled methacholine (3, 5, and 10 mg/ml) was calculated on Day 115 (31), after which the animals were killed, and the left lungs were removed and inflated with 30% formalin/PBS for 10 minutes. Lungs were fixed in 4% formaldehyde solution before immunohistochemical analysis.

Immunohistochemical Detection of Angiogenesis in Mouse Lung Samples

Paraffin-embedded lung tissue sections were stained for vWF (rabbit antimouse vWF; Santa Cruz Biotechnology Inc.). Four slides per lung were selected at random, and the entire section was imaged. Blood vessel numbers per field, the area of each vessel, and the total area of

each field were determined. Total blood vessel area was divided by the total area of the section and by the number of blood vessels in that field.

Immunohistochemical Detection of VEGF or IL-13 in Mouse Lung Samples

Paraffin-embedded lung tissue sections were stained for VEGF (goat antimouse VEGF; Sigma Aldrich) or IL-13 (goat antimouse IL-13; Santa Cruz Biotechnology). Trypan blue was used to quench autofluorescence (32). Image analysis was performed using Quantity One (BioRad) to measure the staining intensity within a given area that was reported as the staining volume. The ratio of the VEGF or IL-13:isotype control staining was calculated for three regions (VEGF) or 8 to 10 segments (IL-13) per airway.

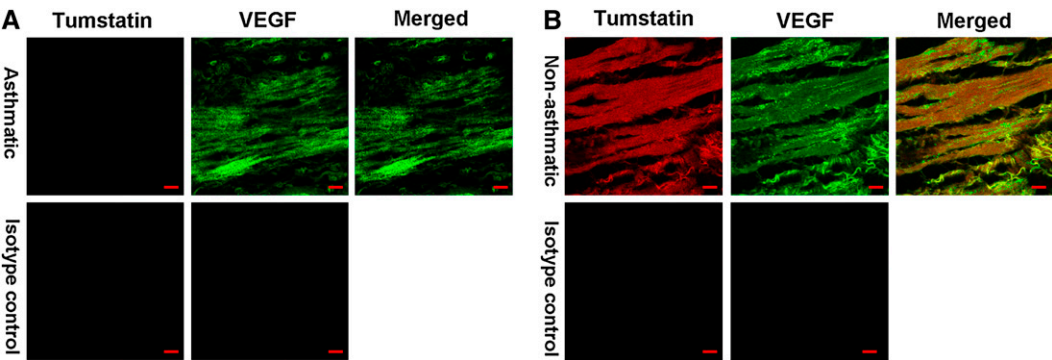


Figure 2. Immunofluorescent detection of colocalization of tumstatin and vascular endothelial growth factor (VEGF). Paraffin-embedded airway sections from (A) subjects with asthma and (B) subjects without asthma were double stained for tumstatin (red fluorescence) and VEGF (green fluorescence) or the appropriate isotype controls. Images are representative of results obtained from three subjects with asthma and three subjects without asthma.

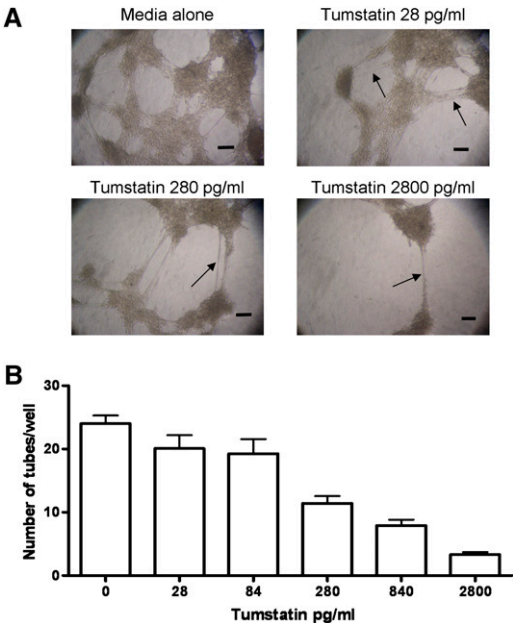


Figure 3. Tumstatin reduces pulmonary endothelial cell tube formation *in vitro*. (A) Primary pulmonary endothelial cell tube formation was assayed using a commercial tube formation kit. Endothelial cell tubes are indicated by the arrows. Scale bars represent 200 μ m. All the images are representative of results observed from six primary pulmonary endothelial cell lines. (B) Primary pulmonary endothelial cells ($n = 6$) were exposed to recombinant tumstatin (28–2800 pg/ml), and tube formation was assessed. The number of tubes formed per well was determined by manual counting. Data are expressed as mean $\pm$ SEM. * $P < 0.05$ versus 0 tumstatin. # $P < 0.01$ versus 0 tumstatin.

Immunohistochemical Detection of Airway Inflammation in Mouse Lungs

Paraffin-embedded mouse lung tissue sections were stained using Alcian Blue (pH 1) (eosinophils) (33) and the Periodic Acid Schiff

method (mucus producing cells) (34–36) as described previously. A blinded average score for each cell type was calculated from 10 high-power fields, and the results were subject to ANOVA.

Full details of the *in vivo* and *in vitro* protocols are available in the online supplement.

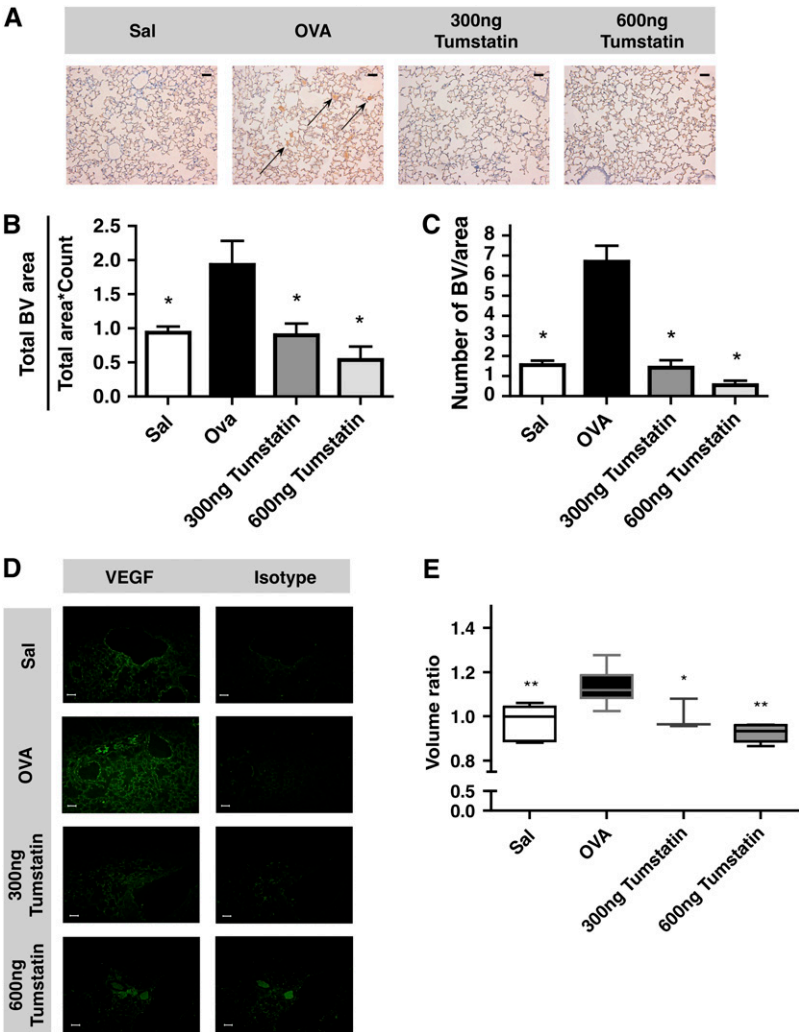


Figure 4. Tumstatin reduces angiogenesis and vascular endothelial growth factor (VEGF) accumulation *in vivo* in a mouse model of chronic ovalbumin (OVA)-induced allergic airways disease. (A) Detection of angiogenesis (von Willebrand factor–positive staining) in paraffin-embedded lung sections from groups receiving saline (Sal), OVA, OVA and 300 ng tumstatin or OVA and 600 ng tumstatin using diaminobenzidine (DAB) (brown). Blood vessels are indicated by arrows. (B) The total area of each field was measured, and the ratio of the number of blood vessels within this area was calculated. (C) The mean blood vessel area was determined by dividing the total blood vessel area by the number of blood vessels in that field normalized to the total area of the section. (D) Paraffin-embedded airway sections were stained with an antibody against VEGF or an isotype control. Green represents specific staining. (E) The staining intensity within the total area on each section was calculated (reported as the volume ratio). Images are representative of results obtained from (A–C) four mice per group or (D and E) from six mice in the saline and OVA groups and two sections from each of three mice in the 300 ng tumstatin and 600 ng tumstatin groups. Data are expressed as mean $\pm$ SEM. (B and C) * $P < 0.02$ compared with OVA. (E) * $P < 0.05$ and ** $P < 0.01$ compared with OVA. Scale bars represent 10 μ m.

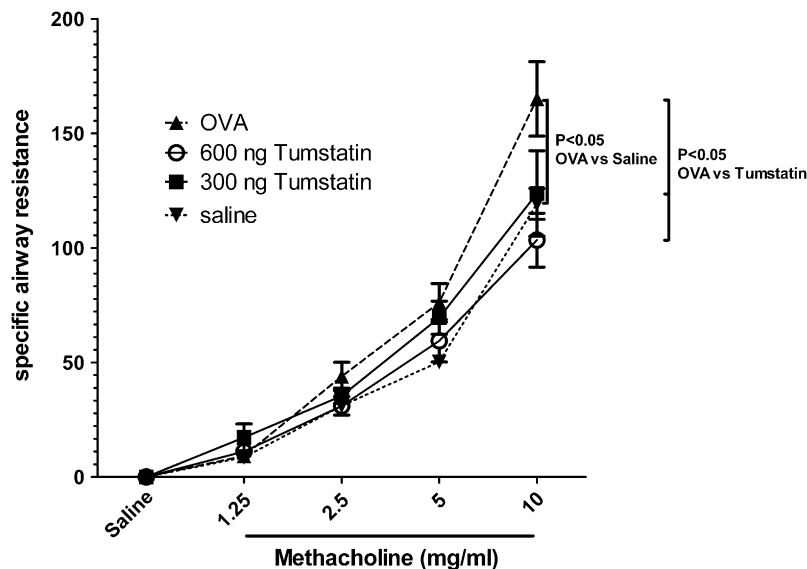


Figure 5. Tumstatin inhibits the development of airway hyperresponsiveness in ovalbumin (OVA)-induced allergic airways disease. Airway resistance was measured using whole-body plethysmography to increasing concentrations of inhaled methacholine. Data are expressed as mean $\pm$ SEM from four mice per group.

RESULTS

Increased Angiogenesis in the Airways of Subjects with Asthma

We confirmed the presence of angiogenesis in tissue obtained from eight of the subjects with asthma used in this study as an increase in the number of CD31 and vWF-positive blood vessels compared with tissues from patients without asthma ($n = 8$) (data not shown).

Collagen IV $\alpha 3$ NC1 Domain (Tumstatin) Is Markedly Reduced in the Airways of Subjects with Asthma

All sections derived from patients with asthma ($n = 5$) collected from formalin-fixed archived tissue were negative for the collagen IV $\alpha 3$ chain NC1 domain (tumstatin) and positive for the remaining α chain NC1 domains (Figure 1A). The non-asthmatic sections ($n = 4$) were positive for all six collagen IV α chain NC1 domains. To confirm that this was not a feature of archived tissue, bronchial biopsies obtained from live volunteers with and without asthma ($n = 6$) were stained for the collagen IV $\alpha 3$ NC1 and $\alpha 5$ NC1 domains. The $\alpha 5$ NC1 domain was used as a comparative control because it showed consistent staining in all tissues stained previously. All six biopsy sections from subjects with asthma were negative for tumstatin and positive for the collagen IV $\alpha 5$ NC1 domain, and the four biopsy sections from subjects without asthma were positive for tumstatin and the collagen IV $\alpha 5$ NC1 domain (data not shown). To ensure the marked reduction of tumstatin was not a general feature of chronic respiratory disorders, tissue sections from patients with chronic obstructive pulmonary disease (COPD) ($n = 8$), cystic fibrosis ($n = 4$), and bronchiectasis ($n = 1$) were examined, and tumstatin was found to be present in every case (Figure 1B).

To confirm decreased tumstatin levels, we performed dot blots on pooled samples of tissue derived from subjects with asthma ($n = 4$) and subjects without asthma ($n = 4$). Levels of tumstatin were barely detectable in the asthmatic tissue, and those in nonasthmatic tissue were approximately 18.5-fold higher (Figures 1C and 1D).

Tumstatin and VEGF Are Colocalized in Nonasthmatic Airways

VEGF was expressed in asthmatic airways and in particular in the ASM bundles. Tumstatin and VEGF were found to be colocal-

ized in the airway sections from subjects without asthma ($n = 3$) (Figure 2). Colocalization was clearly observed in the ASM bundle and elsewhere in the tissue. These experiments confirmed the marked reduction of tumstatin in asthma-derived tissue.

Tumstatin Detected in Asthmatic Serum and BALF

Densitometric evaluation of dot blots of serum and BALF samples showed no significant difference in the levels of tumstatin between subjects with asthma, pre- and post-steroids, and subjects without asthma (Figure E2).

Tumstatin Inhibits Primary Pulmonary Endothelial Cell Tube Formation

We demonstrated for the first time that tumstatin inhibits primary pulmonary endothelial cell tube formation in a dose-related manner. Tumstatin at concentrations of 28 and 84 pg/ml resulted in small, but statistically significant, reductions in the formation of tubes ($P < 0.05$; $n = 6$; repeated measures one-way ANOVA with Dunnett's post test). However at a concentration of 280 pg/mL approximately 40% of the tube formation was inhibited. This inhibition was further increased to 60 and 75% when cells were treated with 840 and 2,800 pg/ml, respectively (Figure 3). The number of endothelial cells in the wells was also reduced in proportion to the amount of tumstatin to which the cells were exposed. The tube formation assay is an *in vitro* method for examination of angiogenesis; however, it is limited because the maturation of blood vessels cannot be assessed. Therefore, we also performed a 3D matrigel experiment, which allows sustained blood vessel growth and the investigation of sprouting. Tumstatin was added to this assay at 2,800 pg/ml, and complete inhibition of tube formation and sprouting was demonstrated (data not shown).

Tumstatin Peptides Inhibit Primary Pulmonary Endothelial Cell Proliferation

We also studied the effects of tumstatin and the tumstatin active component peptides T3 and T7 (28) on the proliferation of ASM cells from individuals with and without asthma as well as pulmonary endothelial cells and fibroblasts from individuals without asthma. Tumstatin, and its active component peptides, did not inhibit the proliferation of ASM cells and fibroblasts ($P < 0.05$; $n = 3$ for both) but did prevent pulmonary endothelial cell proliferation (T3: 74.3 ± 5.8 , 69.0 ± 3.0 , 78.3 ± 2.1 ; T7: $71.3 \pm$

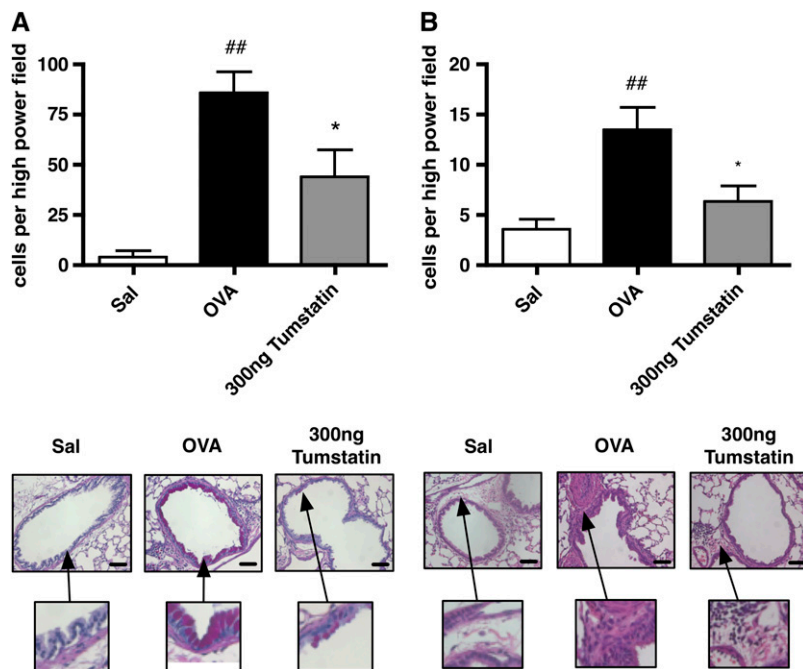


Figure 6. Tumstatin inhibits the development of airway inflammation in chronic ovalbumin (OVA)-induced allergic airways disease. Paraffin-embedded airway sections were stained with Alcian Blue or Periodic Acid Schiff (PAS) for the detection of (A) eosinophils or (B) mucus-producing cells, respectively. Images are representative of results obtained from six mice in saline and four mice in the OVA and the OVA and 300 ng tumstatin groups. Scale bars represent 10 μ m. Cells were identified morphologically and enumerated from 10 high-power fields. Data are expressed as mean $\pm$ SEM from six mice in saline and four mice in the OVA and 300 ng tumstatin groups. * P < 0.05 compared with OVA. $^{##}P$ < 0.05 compared with saline. Sal = saline.

7.8, 56.2 ± 9.3 , $59.6 \pm 10.5\%$ of control on Days 3, 7, and 9, respectively; T3: P < 0.01, n = 6 compared with 5% FBS on all days; T7: P < 0.01, n = 6 compared with time-matched vehicle control on Days 7 and 9 [two-way ANOVA]) (data not shown).

Tumstatin Prevents Angiogenesis and VEGF Production in a Mouse Model of Chronic OVA-induced Allergic Airways Disease

To obtain further insights into the role of tumstatin in the regulation of angiogenesis during allergic airway inflammation, we used a mouse model of chronic ovalbumin (OVA)-induced AAD (30). OVA sensitization and subsequent inhalational exposure induced angiogenesis. Increased numbers of blood vessels were observed in airway sections from OVA-challenged (n = 4) compared with saline-treated (n = 4) groups (Figures 4A–4C).

Treatment of mice that were chronically exposed to OVA with tumstatin (300 ng or 600 ng per 20 g body weight; n = 4 per group) significantly inhibited the development of angiogenesis with a marked reduction in the number of new blood vessels

compared with the untreated OVA-challenged group (P < 0.002; one-way ANOVA) (Figures 4A–4C).

Tumstatin treatment reduced the level of VEGF detected in the airways. Groups that were chronically exposed to OVA exhibited increased intensity of staining for VEGF, which was reversed in animals that were treated with tumstatin (600 ng per 20 g body weight) (Figures 4D and 4E). Levels of VEGF were also reduced in groups treated with 300 ng per 20 g body weight, although this result was not statistically significant.

Tumstatin Improves Airway Hyperresponsiveness in a Mouse Model of Chronic OVA-induced Allergic Airways Disease

Groups chronically exposed to OVA demonstrated an increase in airway resistance to methacholine at the highest concentration tested (10 mg/ml) in comparison to saline-treated groups (P < 0.01; n = 4 in each group; two-way ANOVA with Bonferroni post test) (Figure 5). Treatment with tumstatin (300 ng or 600 ng per 20 g body weight) attenuated airway

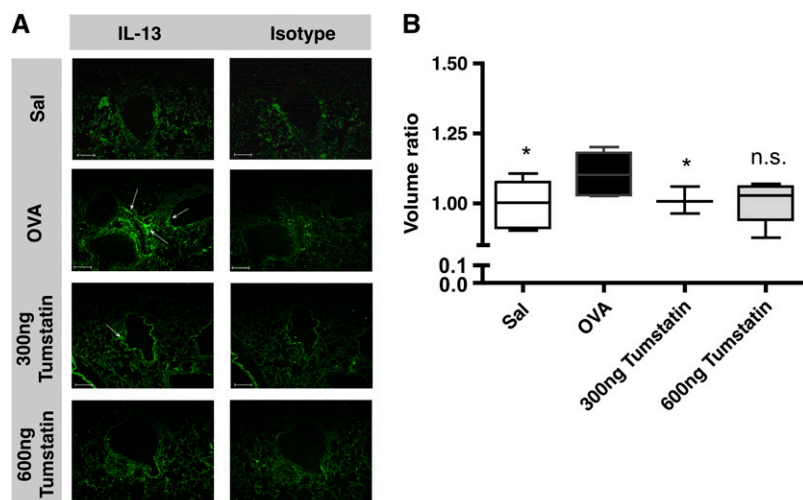


Figure 7. Tumstatin inhibits the accumulation of IL-13 in chronic ovalbumin (OVA)-induced allergic airways disease. (A) Paraffin-embedded airway sections were stained with antibody against IL-13 or an isotype control. Green fluorescence represents specific IL-13 staining. Arrows indicate extracellular IL-13. Scale bars represent 10 μ m. (B) The staining intensity within the total area on each section was calculated (reported as the volume ratio). Images are representative of results obtained from six mice in the saline and OVA groups and two sections from each of three mice in the 300 ng tumstatin or 600 ng tumstatin groups. Data are expressed as mean $\pm$ SEM. * P < 0.05 compared with OVA. Sal = saline.

resistance to methacholine (10 mg/ml) ($P < 0.01$; $n = 4$ in each group; two-way ANOVA with Bonferroni post test) to levels similar to those observed in the saline-exposed (nonallergic) animals (Figure 5).

Tumstatin Inhibits Airway Inflammation in a Mouse Model of Chronic OVA-induced Allergic Airways Disease

To elucidate the mechanism by which treatment reduced airway hyperresponsiveness (AHR), we examined the effect of tumstatin on airway inflammation. As expected, OVA sensitization ($n = 4$) and subsequent inhalational exposure induced allergic inflammation with eosinophilia and increases in the number of mucus-producing cells observed in the airways (Figure 6). Treatment with tumstatin (300 ng per 20 g body weight; $n = 4$) reduced the number of eosinophils and mucus-producing cells in the airways of OVA-challenged animals (Figure 6).

Tumstatin Inhibited IL-13 Accumulation in a Mouse Model of Chronic OVA-induced Allergic Airways Disease

IL-13 staining was significantly increased in lung sections from groups sensitized and challenged with OVA ($n = 6$; $P < 0.05$), particularly in the submucosa, compared with saline-treated control subjects ($n = 6$) (Figure 7A). IL-13 staining was significantly decreased in groups treated with 300 ng per 20 g body weight tumstatin ($n = 4$). A similar pattern was observed in the groups treated with 600 ng per 20 g body weight tumstatin ($n = 4$), although this did not reach significance (Figures 7A and 7B). The areas of increased staining for IL-13 colocalized with areas with increased inflammatory cell influx, comprised of eosinophils (Figure 7) and lymphocytes. Staining was not limited to intracellular regions of the cells (Figure 7, intracellular, *arrow* in 300 ng treatment) but appeared also to be extracellular (Figure 7, *arrows* in OVA).

DISCUSSION

We have demonstrated for the first time a selective marked decrease of the NC1 domain of the collagen IV molecule tumstatin from the airways of patients with asthma. Tumstatin was detected in airway tissue from patients without asthma, and the remaining five collagen IV α chain NC1 domains were present in both groups. The marked reduction of tumstatin was restricted to lung tissue because tumstatin was detected in serum and BALF samples from patients with and without asthma and the levels did not differ. Using primary pulmonary endothelial cells we also demonstrated, for the first time, that tumstatin inhibits pulmonary endothelial cell tube formation in a dose-related manner, identifying a potential role in attenuating angiogenesis. In a murine model of chronic OVA-induced AAD, tumstatin inhibited angiogenesis and the accumulation of VEGF in the airway. Moreover, it attenuated the development of airway resistance, the accumulation of inflammatory cells, and increases in IL-13 levels in the airways.

Our finding of a complete absence of tumstatin in asthmatic tissue was surprising, although a precedent does exist for this in Alport's syndrome, a nephropathy that is associated with absence of the $\alpha 3$ and $\alpha 4$ chains of collagen IV (37). However, this comparison may not be appropriate in that the absence of tumstatin in our study occurred in all subjects with asthma across a wide range of severity, unlike the case with Alport's, a disease in which there are no gradations of severity. To confirm the absence of tumstatin, we therefore used the dot blot technique in addition to immunohistochemistry to assess

tumstatin levels. We were unable to detect tumstatin in asthma-derived tissue using immunohistochemistry, but there was a weak signal in the dot blots. It is likely that the fact that the dot blot was obtained over a longer time frame than that used to detect an immunohistochemical signal could account for the difference in our findings. Nevertheless, the fact that tumstatin was detected by immunohistochemistry in all non-asthmatic tissue and not in any of the asthmatic samples regardless of medication and the finding that quantified levels in the dot blots revealed an 18-fold difference suggest these results by the two methods are consistent. We were surprised that the levels of tumstatin in the BALF and serum were not different in the subjects with and without asthma because tumstatin was absent in asthmatic tissue. Tumstatin is known to be produced in organs other than the lung (e.g., the kidney). It is possible that the levels derived from extrapulmonary sources are much higher than those in the lung, and this could explain why there are no obvious differences in the amount of tumstatin measured in the serum and BALF in the two patient groups.

Angiogenesis is a major feature of the structural changes observed in airway remodeling, and the marked reduction of tumstatin could play a significant role in its development in asthma. Because angiogenesis is necessary to supply nutrients to accumulations of inflammatory cells in chronically inflamed tissue (38), it could be that the chronic inflammation and structural changes observed in asthma are supported by angiogenesis in the airway wall.

Recent asthma research has primarily focused on the role of proangiogenic factors in the induction of angiogenesis. Feltis and colleagues found a relationship between increased levels of VEGF and the number of blood vessels in the airway (39). In addition, in bronchial biopsies from subjects with asthma, blood vessels were more numerous and comprised a greater area of the airway, whereas VEGF was increased in the submucosa (40). TGF- β , the levels of which are increased in the asthmatic airway (12, 14), induces the production and release of VEGF, thereby contributing to vascular remodeling. The role of anti-angiogenic factors in asthma has not been widely examined; however, Asai and colleagues demonstrated that the ratio of VEGF to endostatin in induced sputum from patients with steroid-naïve asthma was increased compared with control subjects and resulted from a change in the levels of VEGF rather than endostatin levels (41).

This is the first report of tumstatin as an angiogenic inhibitor in the airways, although it is known to be a significant suppressor of tumor growth in human renal, prostate, and breast carcinomas (42, 43). Tumstatin is released by the degradation of collagen IV $\alpha 3$ in the basement membrane by specific proteases such as MMPs, in particular MMP9 (10). Circulating levels of tumstatin are decreased in MMP9 deficient mice (10). However, despite the fact that levels of active MMP9 in asthmatic BALF and sputum are reported to be increased (44, 45), we observed no difference in the circulating levels of tumstatin between individuals with and without asthma. Furthermore, the tumstatin that we detected was not cleaved from the whole collagen IV $\alpha 3$ chain, indicating that it was in an inactive state. In contrast to our findings in asthmatic airways, we found that tumstatin was present in the airway tissue of patients with COPD in whom MMP9 levels are known to be high (46–50). This suggests that the relationship between levels of active MMP9 and the presence/absence of tumstatin in the airway tissue is not straightforward. If MMP9 is responsible for the cleavage of tumstatin from the collagen IV molecule, then it would be expected that high levels of MMP9 would result in the disappearance of tumstatin from the airway tissue

of patients with COPD. Because this was not the case in the current study, it is unlikely that the cause of the marked reduction of tumstatin in the asthmatic airway is directly related to MMP9 levels. Many proteases present in the asthmatic airway could be implicated, and these remain the subject of future studies.

We tested the functional activity of tumstatin in two different model systems. First, the use of human primary pulmonary endothelial cells in tube formation assays enabled us to examine the potential of tumstatin to inhibit angiogenesis in airway cells. We recognize that these cells were not derived from the bronchial circulation, which is the most relevant site for assessing the effects of tumstatin on angiogenesis; nevertheless, the cells were of pulmonary origin and were confirmed as endothelial cells morphologically and immunohistochemically. Second, we used a mouse model of chronic AAD to demonstrate that tumstatin inhibited angiogenesis and reduced the level of VEGF in the airway. These findings were accompanied by improved lung function and reductions in the accumulation of inflammatory cells and IL-13. We would have liked to have further validated our findings in a mouse tumstatin knock-out model; however, the phenotype of tumstatin knock-out mice is markedly different from that of human asthma. Most importantly, when collagen 3 is absent, the expression of other collagens is also disrupted (51). Specifically, collagen IV $\alpha 4$ and $\alpha 5$ are also deficient in the lungs of these mice. Given that no impairment of collagen IV $\alpha 4$ and $\alpha 5$ occurs in human asthma, we think that use of the model is likely to generate spurious data.

IL-13 is a well-studied and thoroughly characterized central mediator in allergic asthma (17–21). The transgenic overexpression of IL-13 in the epithelium, or the instillation of recombinant IL-13 in the airways of mice, is sufficient to induce all of the major hallmark features of asthma, such as mucus overproduction, AHR, and allergic inflammation (21, 52). In our model of chronic AAD, IL-13 expression was found to be significantly up-regulated in groups sensitized and challenged with OVA, particularly in the submucosa and colocalized with the sites of highest inflammatory cell infiltrates. Treatment with repeated doses of 300 ng per 20 g body weight tumstatin significantly decreased overexpression of IL-13 in the lungs of allergic groups, providing evidence for an antiinflammatory action of tumstatin. In the IL-13 knock-out mouse, antigen-induced AHR did not occur or increase mucus production (53, 54). We observed a reduction of AHR to baseline levels after treatment with tumstatin and found reduced mucus production in the airways. Therefore, we suggest that treatment with tumstatin inhibits the infiltration of eosinophils and other IL-13-producing cells, such as activated lymphocytes, into the airways, resulting in decreased levels of IL-13 and inhibition of mucus production and AHR. Whether this is due to direct or indirect effects of tumstatin on inflammatory cells requires further investigation.

Our novel findings demonstrate that the lack of tumstatin in the airways of patients with asthma may be important in the development of the phenotypic characteristics of the disease. The key question is: How does tumstatin alter inflammation and AHR? Although tumstatin has been well characterized as an angiogenic inhibitor, it has also been demonstrated to have antiinflammatory properties in human polymorphonuclear cells (55). In a murine model of diabetic nephropathy, tumstatin inhibited the development of disease, the production of VEGF and its corresponding receptor flk-1, and monocyte/macrophage accumulation (56). In our murine model, peripheral angiogenesis (not in the bronchus) occurred, and this was inhibited after treatment with tumstatin. It could therefore be argued that the

antiinflammatory effects that we have reported are the indirect result of the inhibition of angiogenesis. Most murine models of acute AAD (31) do not have histological evidence of angiogenesis. Mouse models that use chronic low-level allergen exposure do induce the development of airway remodeling that involves angiogenesis (30), as well as airway inflammation and AHR, and are more representative of the chronic airway remodeling, airway inflammation, and AHR that occurs in human asthma (57, 58).

The role of collagen fragments in controlling inflammation in asthma has been explored previously in animal models. The administration of endostatin/Fc to OVA-sensitized mice inhibited AHR; pulmonary allergic inflammation; the production of OVA-specific IgE; and expression of CD31, mRNA, and markers of lung inflammation (59). In addition, a naturally occurring multifunctional antiangiogenic agent (neovastat, shark collagen) inhibited airway inflammation and AHR in a murine model of AAD (60). Both these studies produced results that show the protective effects of the antiangiogenic inhibitors endostatin and neovastat using murine models of AAD in which angiogenesis did not occur. Our data therefore suggest that the mechanism underlying the inhibition of inflammation and AHR by tumstatin could be separate from, but enhanced by, the inhibition of angiogenesis.

The ability of tumstatin to inhibit hallmark features of asthma suggests that tumstatin deficiency may play a role in remodeling and alterations in lung function. In a recent case report, a patient with concomitant severe persistent asthma who was treated for rheumatoid arthritis with an angiogenic inhibitor (MEDI-522) had significant and substantial improvements in lung function during the trial (61), a finding that supports the notion that the increased vascularity in asthma has functional relevance. Collectively, our data in the airways in combination with the understanding of the effects of tumstatin in other organ systems suggest that tumstatin may be a novel therapeutic approach to the treatment of angiogenesis and remodeling in asthma.

Conflict of Interest Statement: J.K.B. received more than \$100,001 from the Cooperative Research Centre for Asthma and Airways in sponsored grants and has a patent received or pending from Cooperative Research Centre for Asthma and Airways. S.B. received more than \$100,001 from the Cooperative Research Centre for Asthma and Airways in sponsored grants and holds a received or pending patent from the Cooperative Research Centre for Asthma and Airways. L.M.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.W. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. J.Y.L. is an employee of GlaxoSmithKline, which commenced September 2009, receiving \$10,001–\$50,000 in compensation; she has received a \$1,001–\$5,000 Ph.D. scholarship from the CRC for Asthma and Airways and up to \$1,000 for a patent submitted for working relating to fibulin-1 from the CRC for Asthma and Airways. K.G. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. P.M.H. has a patent received or pending from Newcastle Innovation for novel treatments for asthma and Cortecs/Provalls for novel vaccine targets; none are related to the subject matter of the current manuscript. P.M.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. N.G.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. P.S.F. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. J.L.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. B.G.O. received \$10,001–\$50,000 in industry-sponsored grants from MSD to explore the role of leukotrienes in airway remodeling (ISP).

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Reduction of Tumstatin in asthmatic airways contributes to angiogenesis,
inflammation and hyperresponsiveness

Online Repository

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METHODS

Study population

The study was approved by the Ethics Review Committee of the South West Sydney Area Health Service, Royal Prince Alfred Hospital and The University of Sydney human research ethics committee. All subjects provided written informed consent. The majority of tissue studied was obtained via bronchoscopy, or from surgical tailings / explanted lungs with the exception of one tissue from an asthmatic donor which was obtained at post mortem. Participants were defined as healthy volunteers if they had no preexisting lung disease, normal lung function and no airway hyperresponsiveness, not currently smoking and less than five pack years in total. Asthmatic biopsies, serum, bronchoalveolar lavage fluid (BAL-f) and airway smooth muscle cells (ASM) were obtained from individuals with mild - moderate atopic asthma, according to GINA guidelines (Global Initiative for Asthma, Global Strategy for Asthma Management and Prevention, NIH Publication, 2005). Patients were included if they had asthma symptoms in the preceding 12 months, a positive mannitol/methacoline bronchial provocation challenge test and fewer than 5 pack year history of smoking. Patients were excluded if their baseline forced expiratory volume in the first second (FEV₁) was less than 60% predicted. Some samples were collected prior to and after 7 weeks of treatment with either low dose fluticasone (200 µg [two 50 µg inhalations twice daily, 12 hours apart]) or high dose inhaled fluticasone (1000 µg [two 250 µg inhalations twice daily, 12 hours apart]) delivered through a metered dose inhaler via a large volume spacer. Non-asthmatic individuals had no history of asthma or other lung disease, had normal baseline spirometry (at least 80% predicted), negative mannitol/methacoline bronchial challenge test and no significant

bronchodilator reversibility (12% and 200 mLs improvement in FEV₁). Medication and smoking history was not available for all patients in this study.

Collection and preparation of samples

Tissue specimens were either fixed in formalin and embedded in paraffin or fresh frozen. Biopsies and BAL-f were obtained by means of flexible fibreoptic bronchoscopy performed under local anesthetic with conscious sedation in accordance with the Royal Prince Alfred Hospital Respiratory Medicine Department protocol and the evidence based guideline published by the Thoracic Society of Australia and New Zealand (updated in 2001). BAL-f was collected via normal saline lavage of the segmental airways and alveolar spaces (bronchoalveolar lavage). Endobronchial biopsies of the mucosal lining of the airway were obtained from 4-6th order bronchi. Bronchoalveolar lavage was performed by the instillation of 0.9% sodium chloride solution (4 successive aliquots of 60 mLs, total 240 mLs) warmed to 37°C into the right middle lobe which was then aspirated for collection in a sterile glass bottle (chilled to prevent cellular adhesion to the glass). Five - six superficial biopsies of the bronchial mucosa were obtained using biopsy forceps (Pentax, 1.8mm, cupped) from the carina of the basal segments and subsegments of the right lower lobe. Two - three bronchial biopsies were placed in HBSS for immediate dissection of the airway smooth muscle bundles for culture. Biopsy samples were frozen in OCT and stored at -80°C. ASM cells were isolated as previously described (E1), briefly, bronchial airways were dissected from the surrounding parenchyma and cut longitudinally. With the use of a dissecting microscope, the epithelium was removed with fine forceps in order to expose the smooth muscle bundles. The smooth muscle bundles were dissected free from the surrounding tissue and placed in a sterile tube containing

Human pulmonary endothelial cells were isolated from explanted lungs. Blood vessels were dissected free from surrounding tissue, cut longitudinally and then diced into approximately 5 mm² segments. Blood vessel segments were then placed in digestive buffer, (1 mg (250 U)/ mL type 2 collagenase, 1 U/ mL dispase, 10 mg/ mL BSA) and incubated at 37°C for 10 min with agitation every 2 min. The supernatants were harvested and replaced with fresh digestive mixture every 10 min for a total of 50 min. Ten % FBS (v/v) was added to the supernatants before centrifugation at 200 g for 5 min. The cells were washed with culture medium containing 10% FBS, 10 µg/ mL endothelial cell growth supplement, 20 U/ mL heparin and 2% antibiotics, and re-centrifuged at 200 g for 5 min before being placed in a 75 cm² vented tissue culture flask (Becton & Dickinson (BD)) containing 10ml of culture medium, and incubated for 1 h at 37°C 5% CO₂. Unattached cells were harvested, centrifuged at 200 g for 5 min and placed in a new 75 cm² vented tissue culture flask (BD) (pre-coated with 0.2% (w/v) gelatin (Sigma)) containing 10 mL of culture medium. The cells were then maintained at 37°C in a humidified CO₂ incubator (5% CO₂ in air).

Fibroblasts were isolated from explanted lungs and lung resections. The subpleural parenchyma was diced into 2 mm³ pieces and approximately 5 - 7 pieces were seeded onto pre-wetted (DMEM) 25 cm² cell culture flasks. Cells were maintained in

DMEM (Invitrogen) supplemented with 10% FBS, 1% antibiotics. Flasks were then placed in a humidified CO₂ incubator (5% CO₂ in air) and maintained at 37°C.

Immunohistochemical detection of angiogenesis in human samples

Paraffin embedded airway sections from asthmatic and non-asthmatic individuals were stained for CD31 and von Willebrand factor (vWF). Sections were de-paraffinised and re-hydrated through graded alcohol, before being blocked with a peroxidase blocking agent (DakoCytomation) for 5 min and then washed with PBS. Primary antibodies, goat anti-human CD31 (PECAM-1) (Santa Cruz Biotechnology Inc) [1 µg/ mL] and rabbit anti-human vWF (Santa Cruz Biotechnology Inc) [1 µg/ mL], were added and incubated at room temperature for 1 h. The sections were washed in PBS and incubated with the secondary antibodies, anti-mouse horseradish peroxidase (HRP) (DakoCytomation) and rabbit anti-goat HRP (DakoCytomation), at room temperature for 1 h. After washing in PBS for 5 min the substrate chromogen, liquid 3,3'-diaminobenzidine (DAB) (DakoCytomation), was added and the section incubated for 5 min at room temperature. Following washing in distilled water for 5 min sections were mounted using an aqueous mounting medium (Faramount, DakoCytomation) and coverslipped. The sections were imaged on an Olympus BX51 microscope and processed using Leica imaging software IM1000 (Leica).

Collagen IV α 1- α 6 non-collagenous domain detection

Paraffin embedded tissue sections of bronchial rings from asthmatic and non-asthmatic individuals were stained for the six collagen IV α chain NC1 domains. Sections were de-paraffinised and re-hydrated through graded alcohol. Blocking serum was then added to the sections (10% non-immune horse serum) for 20 min at

room temperature. Without rinsing, either primary antibodies (collagen IV alpha 1-6 NC1 a kind gift from Dr Sado Shigei Medical Research Institute, Okayama, Japan [1 ng/ mL]) or isotype control antibody (Rat IgG, Jackson ImmunoResearch [1 ng/ mL]) were added to the sections and incubated for 1 h at room temperature. Sections were then washed with PBS and a goat anti-rat FITC-conjugated secondary antibody (MP Biomedicals [1 ng/ mL]) was added, and incubated for 30 min at room temperature. Sections were then rinsed with PBS and mounted using vectashield mounting media (Vecta Laboratories). Bronchial rings from individuals with COPD, cystic fibrosis and bronchiectasis were stained for collagen IV α 3 NC1. Images were taken on an Olympus BX51 fluorescence microscope and analyzed using Leica imaging software IM1000 (Leica).

In addition, sections from fresh frozen biopsies from asthmatic and non-asthmatic individuals were also stained for collagen IV α 1, 3 and 5 NC1 as above, except frozen sections were thawed and placed in PBS for 5 min. The de-paraffinising step was omitted.

Preabsorption with tumstatin

Nitrocellulose membrane was cut to size and pre-wet using PBS for 10 minutes. BAL fluid samples (50 μ L) were loaded into each slot (no dilution) in duplicate. Tumstatin (Weiss Lab) was loaded at concentrations from 0.75 – 0.047 μ g . A vacuum was applied until the samples had gone through. The membrane was washed three times with PBS. The membrane was blocked in 5% skim milk/PBS for 1 hour at room temperature. To investigate the specificity of the antibody, the mouse anti-tumstatin (Weiss lab) antibody (1:100) was preincubated with either PBS or tumstatin (20 μ g) in 1% skim milk for 1 hour at room temperature. The membrane was incubated with the

primary antibody +/- tumstatin overnight at 4°C with orbital shaking. The membrane was then washed three times with PBS and a goat anti mouse (santa cruz sc2005 1:1000) secondary antibody added, and incubated for 2 hours at room temperature. After washing three times, the membrane was visualised using supasignal west dura extended duration substrate kit. The image was captured using a Kodak image station is4000 mm and Kodak mi (version 4.0.5) software.

Co-localization of tumstatin and VEGF

Paraffin sections from asthmatic and non-asthmatic individuals were stained for tumstatin and VEGF. Bronchial sections were de-paraffinised and placed in water for 5 min. Sections were blocked in 10% horse serum for 20 min at room temperature and blotted dry. Primary antibodies, rat anti-human collagen IV $\alpha 3$ NC1 (Shigei Medical Research Institute, Okayama, Japan) at 1 ng/ mL and rabbit anti-human VEGF₁₆₅ (Chemicon International) at 1ng/ml, were combined and added to the sections. Isotype controls, Rat IgG, (Jackson ImmunoResearch [1 ng/ mL]) and rabbit IgG (R&D Systems [1 ng/ mL]) were added in the same manner as primary antibodies. Sections were incubated at room temperature for 1 h. Sections were then washed in PBS for 5min and secondary antibodies were added. Alexa fluor 633 anti-rat [1 ng/ mL] and Alexa fluor 488 anti-chicken [1 ng/ mL] (Molecular Probes) were added to the sections and incubated for 30 min at room temperature. Sections were then washed in PBS for 5 min and mounted using Dabco anti-fade mounting medium (Sigma). Images were acquired using the Zeiss LSM 510 Meta confocal microscope.

Dot Blot

The amount of collagen IV $\alpha 3$ NC1 domain in the serum and BAL-f of asthmatic individuals, pre and post steroids, and non-asthmatics was compared by dot blot (Slot Blot manifold, PR 648, GE healthcare).

BAL-f and serum samples were concentrated (centricon with ultracel YM-100 Millipore Corporation) and both fractions were analyzed by dot blot analysis with anti-tumstatin, visualized using chemiluminescence (West Dura Extended, Pierce Biotechnology) and quantified using a Kodak *ds* image station IS4000 MM.

Recombinant tumstatin was used as a positive control.

BAL-f and serum samples were concentrated using centrifugal filter devices with a 100kD pore size (Millipore Corporation). Samples from above ($>100\text{kD}$) and below ($<100\text{kD}$) the filter were collected and analyzed by dot blot. Nitrocellulose membrane (Hybond-ECL 0.45 μm pore size, Amersham) was washed in tris buffered saline with 0.05% Tween (T-TBS) for 5 min before use. Fifty μl of serum or BAL-f was loaded into each slot and the vacuum applied until samples were completely evacuated.

Recombinant tumstatin (Recombinant peptide, NC1 domain from bovine collagen IV, Weislab) was used as a positive control. The membrane was then washed twice with T-TBS, which was extracted with the aid of the vacuum, before being removed from the blotting apparatus and blocked in 5% BSA for 1 h at room temperature. Primary antibody, mouse anti-human collagen IV NC1 domain of the $\alpha 3$ chain (Wieslab) was added at a 1:1200 dilution in 0.1% BSA /T-TBS respectively and incubated for 1 h at room temperature. The membrane was then washed three times with T-TBS and, incubated with secondary antibody (rabbit anti-mouse HRP at a 1:2000 dilution in 0.1% BSA/T-TBS (Dakocytomation, Abcam respectively), for 30 min at room temperature. After an initial wash with T-TBS for 15 min the membrane underwent a

further 2 washes of 5 min each before being visualized, imaged and densitometry calculated using a Kodak *ds* image station IS4000 MM, following the addition of chemiluminescent substrate (West Dura Extended, Pierce Biotechnology).

The blot was blocked with 5% milk in TPBS for 2hrs at RT. The primary AB was diluted 1:100 in 5% milk/TBS and incubated at 37°C for 2h. The secondary antibody was diluted 1:250 in TBS and applied for 1h at RT. Picture was taken at a 2x2 binning for 25 sec. Col IV $\alpha 1$ and $\alpha 2$ were used as a loading control

Endothelial Cell Tube Formation

Endothelial cell tube formation in the presence and absence of tumstatin was assessed with a commercial tube formation assay (BD BioCoat Angiogenesis system- endothelial cell tube formation, BD Biosciences). Fifty μ l of endothelial cells were seeded onto the 96 well plate at 4×10^5 cells/ mL in 10% FBS (JRH Biosciences) HAMS Nutrient F-12 (JRH Biosciences) with endothelial cell growth supplement (ECG, BD Biosciences). Tumstatin was added in duplicate wells in increasing concentrations from 0-2800 pg/ mL. Following overnight incubation (37°C, 5% CO₂) tube formation (angiogenesis) was visualized using an inverted light microscope. Images were taken using a digital camera (Olympus Camedia C-4000 Zoom digital compact camera 4.0 megapixel). The number of tubes/well were counted and statistical analysis (one way ANOVA with Dunnett's post test) was performed.

3D Matrigel

12 well plates were coated with 400 μ l of Matrigel (BD Biosciences) and incubated at 37°C for 1 h. Endothelial cells were seeded onto the 12 well plates at 4×10^4 cells/ mL in 10% FBS/F-12 with ECG in the presence of VEGF 100 ng/ mL, angiopoietin-1

(Ang-1) 100 ng/ mL, ephrin-B2 (Eph-B2) 300 ng/ mL (all from R&D Systems) and 5% fibrin (Sigma-Aldrich). Tumstatin was added at 2800 pg/ mL in duplicate wells at time 0 and d2. Plates were incubated at 37°C, 5% CO₂ for 7 d. Cells were restimulated every 48 h. Images were taken using a digital camera (Olympus Camedia-4000 Zoom Digital Compact Camera 4.0 megapixel) every day for 7 d.

Western Blotting

A non-reducing western blot using an antibody against tumstatin was performed. Briefly, serum samples were used at a 1:500 dilution. Twenty µL of sample was added to 20 µL of loading buffer, (20% glycerol, 1M Tris HCL pH 6.8, bromophenol blue) and loaded onto pre-cast gradient gels, 4%-20%, (Biorad) and the gels were run at 100 Volts for 75 min. Proteins were then transferred onto polyvinylidene difluoride membranes for 90 min at 30 Volts. Membranes were blocked for 1 h at room temperature with 5% BSA/TBS and primary antibody against tumstatin (Wieslab) added at a 1:2000 dilution and incubated overnight at 4°C with orbital shaking. Membranes were then washed with T-TBS and incubated with rabbit anti-mouse HRP (Dakocytomation) for 1 h at room temperature. Proteins were visualized by the addition of chemiluminescent substrate (West Dura Extended, Pierce), and imaged using the Kodak *ds* image station IS4000 MM.

Inhibition of proliferation

ASM cells from asthmatic and non-asthmatic individuals and fibroblasts and primary pulmonary endothelial cells from non-asthmatic individuals, were seeded in 96 well plates at 1×10^4 cells /cm², ASM and fibroblast cells in DMEM containing 5% FBS, endothelial cells, in F-12 10% FBS, for a period of 24 h. The cells were then washed

three times using HBSS and placed in DMEM/F-12 containing 0.1% insulin transferrin and selenium (ITS) (Invitrogen) for 24 h. Following washing with HBSS (3 times), growth medium was added to the cells. The synthetic tumstatin derived peptides T3 (Leu-Gln-Arg-Phe-Thr-Thr-Met-Pro-Phe-Leu-Phe-Cys-Asn-Val-Asn-Asp-Val-Cys-Asn-Phe) dissolved in water or T7 dissolved in 4% acetonitrile (Thr-Met-Pro-Phe-Leu-Phe-Cys-Asn-Val-Asn-Asp-Val-Cys-Asn-Phe-Ala-Ser-Arg-Asn-Asp-Tyr-Ser-Tyr-Trp-Leu) (Phoenix pharmaceuticals) were added at 4.5 μ M to some wells. Acetonitrile (4%) was used as a vehicle control. The peptides were replaced after the initial 24 h and then after every 48 h. Proliferation was assessed using an MTT assay at d 3, 7 and 9. Briefly 10 μ L of MTT was added to each well for 5 h at 37°C followed by the addition of 100 μ L of filtered 10% SDS in 0.01M HCl overnight. The specific absorption of each well was measured at 690 nm (reference) and 570 nm.

Ovalbumin mouse model

A chronic model of allergic airway inflammation was generated as follows. Adult Balb/c mice (6-8 weeks old) were administered OVA on d 0, 7, 14 and 21: (25 μ g OVA and 1 mg aluminum hydroxide in 200 μ L sterile PBS subcutaneously [SC]). On d 26, 29 and 31, 20ng OVA in 50 μ L sterile PBS was administered intranasally (IN), and then twice weekly from d 32 – 115. Some of the mice in the chronic asthma model were administered tumstatin (reconstituted in PBS) IN at either 600 ng or 300 ng per 20 g body weight once a day from d 25-115. Sham treatment was as follows: Adult Balb/c mice (6-8 wks old) were administered 1 mg aluminum hydroxide in 200 μ L sterile PBS SC on d 0, 7, 14 and 21. On d 26, 29 and 31, 50 μ L sterile PBS was administered IN. Twenty ng OVA in 50 μ L sterile PBS was administered IN, twice

weekly from d 32 – 115. The airway resistance to inhaled methacholine (10 mg/ mL) was calculated on d 115, at this time point the animals were sacrificed and the left lung removed and inflated with 30% formalin/ PBS for 10 min. Lungs were then fixed in 4% formaldehyde solution prior to use in immunohistochemical analysis.

The degree of airway hyperresponsiveness was measured to increasing concentrations of inhaled methacholine *in vivo* by measuring changes in transpulmonary resistance (R_L) and dynamic compliance (C_{dyn}) using a supine whole-body plethysmograph (Buxco Electronics Inc) attached to a computer (E2). Mice were anaesthetized with an intraperitoneal injection of ketamine/xylazine and cannulated *via* the trachea with an 18 G metal tube. Mice were mechanically ventilated by a Minivent mouse ventilator at a rate of 120 breaths per min and a tidal volume of 8 mL/ kg. Changes in lung volume were detected by a differential transducer connected to the plethysmograph chamber lumen which measured volume changes due to thoracic expansion with ventilation. A pressure transducer with a port near the tracheal tube measured alterations in tracheal pressure as a function of airway caliber. Aerosolized acetyl- β -methacholine (1.25, 2.5, 5, and 10 mg/ml) in PBS generated by an ultrasonic nebulizer was delivered directly to the lungs via the inspiratory line for 5 min. The peak response to methacholine was compared to the response to saline alone. At the end of the study mice were sacrificed and the lungs were inflated with 30% formalin/ PBS for 10 min.

Immunohistochemical detection of angiogenesis in mouse lung samples

Paraffin embedded lung tissue sections were de-paraffinized, and re-hydrated through graded alcohol. Sections were incubated with peroxidase blocking solution (Dako

Cytomation) for 5 min. Sections were then washed with PBS and primary antibody was added, rabbit anti-mouse vWF (Santa Cruz Biotechnology Inc) at 1:100 dilution, and incubated at room temperature for 1 h. Sections were washed in PBS and incubated with the secondary antibody anti-rabbit HRP (DakoCytomation) at room temperature for 1 h. Sections were washed in PBS for 5 min and incubated with substrate chromogen, liquid DAB (DakoCytomation) for 5 min at room temperature. Sections were then washed in distilled water for 5 min and coverslipped using an aqueous mounting medium (Faramount, DakoCytomation). Images were taken on an Olympus BX51 fluorescence microscope and captured using Leica imaging software IM 1000 (Leica). Four slides per lung were selected at random and the entire section imaged. Blood vessel numbers per field were counted and the area of each vessel measured as well as the total area of each field. Total blood vessel area was divided by the total area of the section and divided by the number of blood vessels counted in that field.

Immunohistochemical detection of VEGF in mouse lung samples

Slides were de-paraffinized and rehydrated in graded alcohol, and washed for 5 min. Slides were blocked in 10% (v/v) non-immune horse serum in PBS for 1 h at room temperature and washed in PBS three times. One hundred μL / section of either a goat anti-mouse VEGF (15 $\mu\text{g}/\text{mL}$ in PBS) (Sigma Aldrich) or a goat IgG isotype control (15 $\mu\text{g}/\text{mL}$ in PBS) (Jackson Immunol Research Laboratories) was added to each section and incubated for 1 h at room temperature. After incubation, slides were washed in PBS three times. The primary antibodies were detected using a FITC-conjugated rabbit anti-goat antibody (11.25 $\mu\text{g}/\text{mL}$ in PBS) (Sigma Aldrich) for 30 min at room temperature in the dark. Slides were washed in PBS three times. To

quench autofluorescence of the sections, 200 μ L/ well trypan blue was added to each sample (E3). Slides were washed in PBS three times prior to being mounted using antifade solution (Sigma Aldrich). Images were taken on an Olympus fluorescence microscope (model BX51) and captured using Leica IM1000 image capture software. The fluorescent threshold was set using the corresponding isotype control. Image analysis was performed using Quantity One (BioRad) to measure the staining intensity within a given area which was reported as the staining volume. The ratio of the VEGF:isotype control staining was calculated for 3 regions per slide.

Immunohistochemical detection of airway inflammation in mouse lungs

Paraffin embedded mouse lung tissue sections de-paraffinized and re-hydrated through graded alcohols to water. Sections were stained using the Alcian Blue, pH 1, method for sulphated mucosubstances (E4) for the detection of eosinophils and the Periodic Acid Schiff's (PAS) Method for Carbohydrates (E5-7) for the detection of mucus producing cells as described previously.

The operator was blinded to treatment and images were captured using an Olympus (BX51) upright microscope and a Leica DC500 digital color camera together with Leica IM1000 image capture software. Lung histology was analyzed as described previously (E8). Briefly, lung section representing the central (bronchi-bronchiole) and peripheral (alveoli) airways were divided into high-power-fields (HPF) according to morphological and physiological features in each of the lung section. Cells were identified morphologically and counted from 10 HPF. An average score for each cell type was calculated from the 10 HPFs. Lung sections from four individual animals in

each treatment group were counted and the results were subject to ANOVA statistical analysis.

Immunohistochemical detection of IL-13 in mouse lung samples

Slides were de-paraffinized before rehydration through graded alcohols to water.

Slides were blocked in 10% (v/v) non-immune horse serum in 0.05% Tween 20 (Amresco) PBS (T-PBS) for 1 h at room temperature. One hundred μ L/ section of either 4 μ g/ mL of primary antibody, goat anti-mouse IL-13 (Santa Cruz Biotechnology), or an isotype ChromPure Goat IgG (Jackson Immuno Research), was added to each section and incubated for 1 h at room temperature. After incubation, slides were washed in T-PBS. The primary antibodies were detected using a 1:400 dilution of anti-goat FITC antibody (Sigma) for 30 min at room temperature in the dark. Slides were washed in T-PBS. Sections were then treated with ice-cold 0.4% trypan blue (Sigma) in T-PBS for 10 min to quench autofluorescence. Slides were then washed with PBS and coverslipped using antifade mountant (Dabco Sigma). Images were taken on an Olympus fluorescence microscope (model BX51) and captured using Leica IM1000 image capture software. The fluorescent threshold was set using the corresponding isotype control. The ratio of the IL-13:isotype control staining was calculated for 8-10 segments per airway.

RESULTS

Tumstatin detected in asthmatic serum and bronchoalveolar lavage fluid.

We then determined whether the marked reduction of tumstatin detected in the airway sections in the asthmatic population was lung tissue specific or whether it was affected by corticosteroid treatment. The presence of tumstatin was assessed in serum and bronchoalveolar lavage fluid (BAL-f) samples from 10 asthmatic patients who

were sampled pre and post treatment with inhaled corticosteroids for 7 weeks, and 7 non-asthmatic individuals (supplemental figure 2A).

Tumstatin is active as an angiogenic inhibitor only when it is cleaved from the collagen IV $\alpha 3$ chain. To determine whether tumstatin detected in the serum was cleaved, and therefore active, serum samples were assessed using western blots. The whole collagen IV $\alpha 3$ chain at 188 kD rather than the active tumstatin at 28kD was present in both the asthmatic and non-asthmatic serum (supplemental figure 2B). To further confirm our results both serum and BAL-f samples were separated into fractions containing molecules greater than and less than 100kD and analyzed by dot blot for tumstatin. Tumstatin was detected only in the >100kD fraction of both serum and BAL-f from asthmatics, pre and post steroids, and non-asthmatics for all samples tested (supplemental figure 2C).

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Supplementary figure 1. Recombinant tumstatin (0.75 – 0.047 ug/ml), and BAL-f samples from non-asthmatics (n=5), and asthmatics pre (n=6) and post (n=6) steroid treatment were examined by dot blot. Values with no preabsorption are shown on the left and the blots on the right hand side were obtained following pre-absorption of the anti-tumstatin antibody with recombinant tumstatin.

Supplementary Figure 2. **Detection of tumstatin in serum and bronchoalveolar lavage fluid.**

Levels of tumstatin in serum from asthmatics pre (n=10) and post (n=10) steroid treatment and non-asthmatics (n=7) measured by dot blot (A). Individual densitometric values were normalized to the positive control value on each blot and data are expressed as mean $\pm$ SEM. Serum from asthmatics and non-asthmatics analyzed by western blot for tumstatin (B). Recombinant peptide NC1 domain from bovine collagen IV α 3 (tumstatin) was used as the positive control. The image is representative of results obtained from asthmatic n=10 pre and post steroids and non-asthmatics n=7. BAL-f samples from asthmatics pre (n=10) and post (n=10) steroid treatment and non-asthmatics (n=7) were fractionated (<100kD and >100kD) and analyzed by dot blot for the presence of tumstatin (C). Individual densitometric values were normalized to the positive control value on each blot and data are expressed as mean $\pm$ SEM.

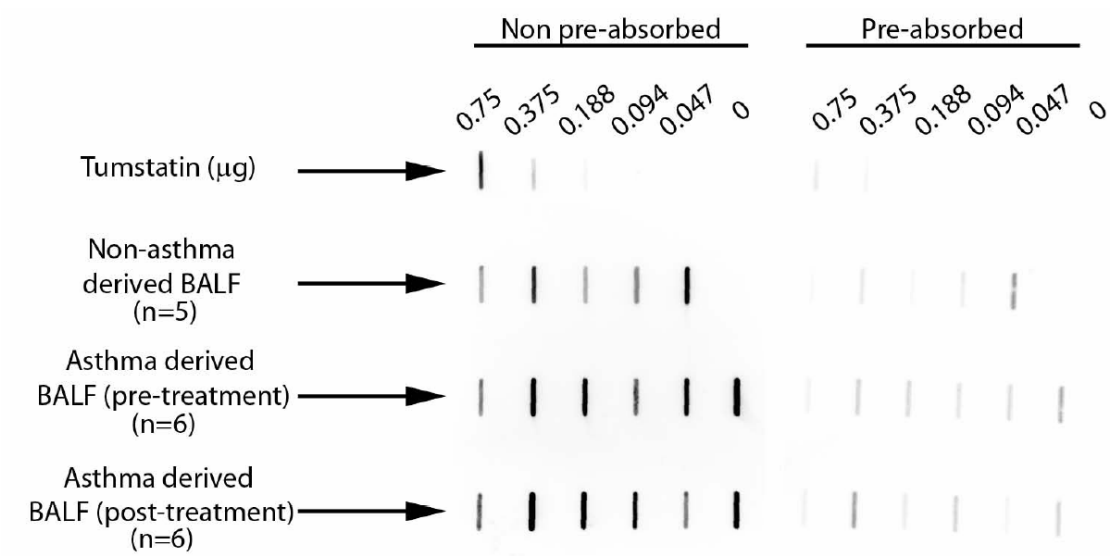
Table 1. Patient demographics

Diagnosis	Age	Sex	Sample	Smoking	Medication	FEV1 % pred	Experiments where used
Asthma	45	M	bronch biopsy	No	ICS + β_2	101	Coll IVa IHC
Asthma	38	M	bronch biopsy	No	ICS + β_2	77	Coll IVa IHC
Asthma	50	F	bronch biopsy	No	ICS, β_2 , oral CS (1/12)	70	Coll IVa IHC
Asthma	20	M	bronch biopsy	No	β_2 agonist	103	Coll IVa IHC
Asthma	60	M	bronch biopsy	No	ICS, β_2 , oral CS (1/12)	94	Coll IVa IHC
Asthma	42	M	bronch biopsy	No	ICS + β_2	88	Coll IVa IHC
Asthma	48	M	explanted lung	No	NA	NA	Coll IVa IHC
Asthma	15	F	explanted lung	No	NA	NA	Coll IVa IHC
Asthma	17	M	explanted lung	No	ICS, β_2 , oral CS (1/12)	64	Coll IVa IHC + co
Asthma	80	M	PM Lung	No	NA	NA	Coll IVa IHC + co
Asthma	33	F	explanted lung	No	ICS + β_2	NA	Coll IVa IHC + co
Bronchiectasis	46	M	explanted lung	NA	NA	NA	Coll IVa IHC
CF	19	F	explanted lung	NA	NA	NA	Coll IVa IHC
CF	22	F	explanted lung	NA	NA	NA	Coll IVa IHC
CF	25	F	explanted lung	NA	NA	NA	Coll IVa IHC
CF	29	M	explanted lung	NA	NA	NA	Coll IVa IHC
COPD	63	F	bronch biopsy	>15 PY	β_2 only for 4/52	75.5	Coll IVa IHC
COPD	58	M	bronch biopsy	>15 PY	β_2 only for 4/52	64	Coll IVa IHC
COPD	57	F	bronch biopsy	>15 PY	β_2 only for 4/52	39	Coll IVa IHC
COPD	73	M	bronch biopsy	>15 PY	β_2 only for 4/52	59	Coll IVa IHC
COPD	64	M	bronch biopsy	>15 PY	β_2 only for 4/52	80	Coll IVa IHC
COPD	75	M	bronch biopsy	>15 PY	β_2 only for 4/52	40.7	Coll IVa IHC
COPD	67	M	bronch biopsy	>15 PY	β_2 only for 4/52	62	Coll IVa IHC
COPD	68	M	bronch biopsy	>15 PY	β_2 only for 4/52	22	Coll IVa IHC
Healthy Volunteer	43	M	bronch biopsy	ex 3 PY	none	NA	Coll IVa IHC
Healthy Volunteer	22	M	bronch biopsy	no	none	104	Coll IVa IHC
NA (lung donor)	47	M	PM Lung	NA	NA	NA	Coll IVa IHC
Non-Asthma	57	M	surgical tailings	NA	NA	NA	Coll IVa IHC + co
Non-Asthma	53	M	surgical tailings	NA	NA	NA	Coll IVa IHC + co
Non-Asthma	62	M	surgical tailings	NA	NA	NA	Coll IVa IHC + co
Asthma	31	F	bronch biopsy	No	ICS, β_2 , oral CS (1/12)	<60%	Tum dot blot
Asthma	30	M	bronch biopsy	No	β_2 agonist	101	Tum dot blot
Asthma	42	F	bronch biopsy	No	none	NA	Tum dot blot
Asthma	62	M	bronch biopsy	No	ICS + β_2	85	Tum dot blot
Healthy Volunteer	22	F	bronch biopsy	No	none	NA	Tum dot blot
Healthy Volunteer	21	M	bronch biopsy	1 PY	none	88	Tum dot blot
Healthy Volunteer	29	M	bronch biopsy	No	none	80	Tum dot blot
Healthy Volunteer	20	F	bronch biopsy	No	none	98	Tum dot blot
Asthma	21	M	BALf, serum	No	Pre & post 200 ug flut	86	Tum dot blot
Asthma	21	M	BALf, serum	No	Pre & post 200 ug flut	62	Tum dot blot
Asthma	20	M	BALf, serum	No	Pre & post 1000 ug flut	105	Tum dot blot
Asthma	20	M	BALf, serum	No	Pre & post 1000 ug flut	80	Tum dot blot

Asthma	22	M	BALf, serum	No	Pre & post 200 ug flut	82	Tum dot blot
Asthma	27	M	BALf, serum	No	Pre & post 200 ug flut	76	Tum dot blot
Asthma	21	M	BALf, serum	No	Pre & post 200 ug flut	98	Tum dot blot
Asthma	30	M	BALf, serum	No	Pre & post 1000 ug flut	75	Tum dot blot
Asthma	26	M	BALf, serum	No	Pre & post 200 ug flut	98	Tum dot blot
Asthma	22	M	BALf, serum	No	Pre & post 1000 ug flut	100	Tum dot blot
Healthy Volunteer	26	M	BALf, serum	No	none	92	Tum dot blot
Healthy Volunteer	22	M	BALf, serum	No	none	97	Tum dot blot
Healthy Volunteer	33	M	BALf, serum	No	none	125	Tum dot blot
Healthy Volunteer	25	F	BALf, serum	No	none	102	Tum dot blot
Healthy Volunteer	23	M	BALf, serum	No	none	80	Tum dot blot
Healthy Volunteer	19	M	BALf, serum	No	none	104	Tum dot blot
Healthy Volunteer	20	M	BALf, serum	No	none	82	Tum dot blot
emphysema	65	M	explanted lung (E)	NA	NA	NA	tube formation
Primary pulmonary hypertension	44	F	explanted lung (E)	NA	NA	NA	tube formation
emphysema	52	F	explanted lung (E)	NA	NA	NA	tube formation
pulmonary fibrosis	44	M	explanted lung (E)	NA	NA	NA	tube formation + proliferation
graft vs host disease	23	M	explanted lung (E)	NA	NA	NA	tube formation + proliferation
emphysema	26	F	explanted lung (E)	NA	NA	NA	tube formation + proliferation
emphysema	64	F	explanted lung (E)	NA	NA	NA	proliferation
emphysema	55	M	explanted lung (E)	NA	NA	NA	proliferation
emphysema	64	F	explanted lung (F)	NA	NA	NA	proliferation
emphysema	55	M	explanted lung (F)	NA	NA	NA	proliferation
pulmonary fibrosis	44	M	explanted lung (F)	NA	NA	NA	proliferation
carcinoma	74	M	explanted lung (A)	NA	NA	NA	proliferation
carcinoma	75	M	explanted lung (A)	NA	NA	NA	proliferation
donor lung	20	M	explanted lung (A)	NA	NA	NA	proliferation

Abbreviations used, bronch biopsy: bronchoscopic biopsy, PM Lung: post mortem lung, BALf: bronchoalveolar lavage fluid, explanted lung (E): endothelial cells from explanted lungs, explanted lung (F): fibroblasts from explanted lungs, explanted lung (A): airway smooth muscle cells from explanted lungs, ICS: inhaled corticosteroids, β_2 : β_2 agonist, oral CS (1/12): oral corticosteroids once the previous twelve months, β_2 only for 4/52: only β_2 agonist for the previous 4 weeks, Coll IV α IHC: immunohistochemistry for collagen 4 α chains, Coll IV α IHC + co: as before + colocalization, Tum dot blot: Tumstatin dot blot, flut: Fluticasone, NA: not available.

Supplementary Figure 1



Supplementary Figure 2

