

ORIGINAL ARTICLE

Asthma and Rhinitis

Interleukin-17A and vascular remodelling in severe asthma; lack of evidence for a direct role

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Summary

Background: Bronchial vascular remodelling may contribute to the severity of airway narrowing through mucosal congestion. Interleukin (IL)-17A is associated with the most severe asthmatic phenotype but whether it might contribute to vascular remodelling is uncertain.

Objective: To assess vascular remodelling in severe asthma and whether IL-17A directly or indirectly may cause endothelial cell activation and angiogenesis.

Methods: Bronchial vascularization was quantified in asthmatic subjects, COPD and healthy subjects together with the number of IL-17A⁺ cells as well as the concentration of angiogenic factors in the sputum. The effect of IL-17A on in vitro angiogenesis, cell migration and endothelial permeability was assessed directly on primary human lung microvascular endothelial cells (HMVEC-L) or indirectly with conditioned medium derived from normal bronchial epithelial cells (NHBE), fibroblasts (NHBF) and airway smooth muscle cells (ASMC) after IL-17A stimulation.

Results: Severe asthmatics have increased vascularity compared to the other groups, which correlates positively with the concentrations of angiogenic factors in sputum. Interestingly, we demonstrated that increased bronchial vascularity correlates positively with the number of subepithelial IL-17A⁺ cells. However IL-17A had no direct effect on HMVEC-L function but it enhanced endothelial tube formation and cell migration through the production of angiogenic factors by NHBE and ASMC.

Conclusions & Clinical Relevance: Our results shed light on the role of IL-17A in vascular remodelling, most likely through stimulating the synthesis of other angiogenic factors. Knowledge of these pathways may aid in the identification of new therapeutic targets.

KEYWORDS

asthma, basic mechanisms, remodelling

1 | INTRODUCTION

Severe asthma, affecting 5%-10% of asthmatic patients worldwide, results in frequent exacerbations that respond poorly to steroids.¹

Accumulating evidence suggests that bronchial vascular remodelling contributes to the onset, progression and severity of chronic obstructive airway diseases including asthma and chronic obstructive pulmonary disease (COPD).² For example the number of vessels and

the percentage of vascular area are increased in bronchial biopsies of asthmatics compared to control subjects.^{3,4} In addition, vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are elevated in the sputum, bronchoalveolar lavage (BAL) and serum from asthmatics and are correlated with vascular permeability index, a feature that is consistent with the development of immature and leaky blood vessels.^{3,5-7}

Of the factors that may contribute to vascular remodelling in obstructive airway disease, IL-17A warrants consideration. IL-17A plays a central role in severe asthma, a phenotype associated with increased neutrophils in the airways and steroid resistance.^{8,9} Several studies have reported the involvement of IL-17A in airway remodelling, including its ability to promote mucus secretion from human airway epithelial cells,¹⁰ increase migration and contraction of airway smooth muscle cells¹¹ and the production of neutrophilic chemokines and cytokines such as CXCL8 and colony stimulatory factors (G-CSF).¹² Although a role for IL-17A in airway remodelling is well-documented,¹³ its biological effect on lung microvascular endothelial cells and thus its potential contribution to vascular remodelling is uncertain. Recently, it was reported that increased pulmonary microvessel density was dependent on IL-17 in peribronchial tissues of chronically allergen-challenged mice. Such density was exacerbated by intra-airway administration of IL-17A.¹⁴ Additional studies have described IL-17A as pro-angiogenic as it promotes endothelial migration, chemotaxis and in vitro tube formation of microvascular endothelial cells.^{15,16} Despite these studies, the plausibility that IL-17A contributes to remodelling of the bronchial vasculature in severe asthma remains unexplored. Here, we analysed vascular remodelling and number of IL-17A⁺ cells in bronchial biopsies from neutrophilic severe asthmatics compared to mild and moderate asthmatics, COPD and healthy subjects and measured angiogenic factors in the sputum of severe asthmatic subjects. We further performed a series of in vitro experiments to show whether IL-17A might directly promote angiogenesis in lung microvascular endothelial cells, or whether it has the potential to do so indirectly, by stimulating angiogenic factor production by airway structural cells (bronchial epithelial cells, fibroblasts or smooth muscle cells).

2 | MATERIAL AND METHODS

2.1 | Study population

All endobronchial tissue and sputum specimens from adult subjects were obtained from the Fonds de la recherche en santé du Québec Respiratory Health Network Tissue Bank (McGill University Health Centre/Meakins-Christie Laboratories Tissue Bank, Montreal, Canada). All subjects provided written informed consent and the study was approved by an institutional review board (2017-2581). After pulmonary-function tests, the subjects were classified as mild, moderate and severe asthmatic and non-asthmatic healthy controls according to the ERS/ATS guidelines.¹⁷ Severe asthma population was selected to mainly include patients with neutrophilic asthma. Gold III/IV COPD subjects were also included in the study. All COPD patients were diagnosed after spirometry and measurement of the lung diffusing capacity, according to the Global Initiative for Chronic Obstructive Lung Disease guidelines 2006¹⁸ and with at least a 10 cigarette pack-years history. The characteristics of the subjects are summarized in Table 1 and in Table S1 (Appendix S1).

2.2 | Bronchoscopy

Bronchoscopy was performed under mild sedation after seventh month from the beginning of the recruitment. Endobronchial biopsies were obtained from the right lung at various segmental and sub-segmental carinae. Following harvesting of tissue, biopsies were fixed immediately in 10% formalin overnight, processed and embedded in paraffin.

2.3 | Sputum collection

Attempts at sputum induction were performed monthly during each visit, for a total of 13 collections.

Sputum induction was performed as described elsewhere.¹⁹ Briefly, severe asthmatic individuals received inhaled salbutamol (200 µg) before starting the test. Sputum induction was performed using aerosols of increasing concentrations of hypertonic saline

TABLE 1 Clinical characteristics of the subjects included in the study. Data for age, FEV₁ and FVC are expressed as the median (range)

	Healthy	Mild asthmatic	Moderate asthmatic	Severe asthmatic	COPD gold III-IV	P-value
N	14	14	10	13	10	ns
Sex (M/F)	6/7-1N/A	7/7	3/7	8/5	9/1	ns
Age (range) in years	30 (20-60)	31 (22-54)	39 (26-65)	48 (35-68)	70.5 (35-76)	*
Atopic (Yes/No)	6/6-2N/A	11/3-0N/A	9/1	9/4	1/6-2N/A	ns
Smoking history (Smoker/Ex smoker/Non-smoker)	1/2/10-1N/A	0/2/12	0/2/7 1N/A	0/3/10	2/8/0 a	*
FEV ₁ (% predicted) (range)	116 (98-125)	90.5 (69-128)	90.5 (69-115)	59 (40-99)	55 (35-73)	#
FVC (% predicted) (range)	117 (97-130)	97.5 (84-132)	98 (80-115)	73 (56-105)	82.5 (68-108)	#

COPD, chronic obstructive pulmonary disease.

**P* < .01 COPD Gold III-IV vs all other groups; #*P* < .01 comparison between all groups with the exclusion of mild vs moderate asthmatic subjects and severe asthmatic vs COPD Gold III-IV; ns, non-statistically significant. N/A, not available.

solution (0.9%, 3%, and 5%) delivered by an ultrasonic nebulizer for 7 minutes of each concentration. After each period of inhalation, the maximum expiratory flow was measured to exclude significant falls in FEV₁ and saline inhalations were stopped if a fall in the value of greater than 10% occurred. Subjects expectorated into a sterile container, which was processed within 2 hours.

Portions of sputum, free of salivary contamination were selected, dispersed with dithiothreitol, filtered and centrifuged. Aliquots of supernatant were stored at -80°C and then processed by ELISA. The final cell pellet was re-suspended in PBS, and a total cell count of leucocytes and cell viability were determined. The cells were pelleted onto slides using a cytocentrifuge and were fixed in acetone: methanol (60:40) for 7 minutes, air-dried, and kept at -20°C until further analysis. Slides were stained with HEMA 3 STAIN SET (Fisher Scientific) and the differential cell counts were calculated based on a count of 300 cells.

The differential cell count in induced sputum, showing high prevalence of neutrophils in severe asthmatic patients is reported in the Table S2.

2.4 | Immunohistochemistry and measurements

5 μm thick sections cut from paraffin-embedded bronchial biopsies were processed for high-temperature epitope unmasking in 0.01 mol L^{-1} sodium citrate buffer, permeabilized in 0.2% Triton X-100 (Sigma-Aldrich) and blocked in universal blocking solution (Dako). Tissues were immunostained with von Willebrand factor antibody as marker of endothelial cells (no antibody cross-reactivity with lymphatic cells was assessed by double staining with the specific marker LYVE-1; data not shown) or IL-17A (Cell Signaling). Antibodies were detected with biotinylated secondary antibody (Dako), streptavidin-HRP/AB Complex (Dako), and diaminobenzidine chromogen substrate (Dako). For double immunohistochemistry, the detection of IL-17A was preceded by the identification of neutrophils, macrophages, eosinophils and CD4 T cells using antibodies against elastase (Dako), CD68 (Dako), eosinophil cationic protein (ECP) and CD4 (Abcam), respectively. The detection was performed by alkaline phosphatase (Dako) and ImmPACTTM Vector[®] Red (Vector Laboratories). Slides were then counterstained with haematoxylin. Isotype controls were prepared by replacing the primary Ab with a non-specific rabbit Ig at the same concentration.

To avoid observer bias, all slides were coded by 1 observer and analysed by another observer (AP) blinded to the subject group. Total tissues were mapped by microscopy (Olympus BX51; Olympus) using a 20 $\times$ objective lens and analysed by ImagePro software at higher magnification. For each sample, all available non-overlapping high power fields with intact tissue covered by basement membrane were examined. The border of each vessel and the subepithelial area beneath the epithelial basement membrane (excluding mucosal glands and smooth muscle bundles) were manually traced with ImagePro software. All structures internal to the vessel endothelial basement membrane, included the lumen, were considered as vascular area and percentage of vascular area was obtained by dividing total

vascular area by submucosal area examined. Vessel numbers were counted and divided by the submucosal area examined to determine the number of vessels per mm^2 . Similarly, the number of IL-17A⁺ cells were referenced to the submucosal area examined and expressed as IL-17A⁺ cells/ μm^2 . The number of neutrophils and macrophages expressing IL-17A were analysed after double immunohistochemistry and expressed as % over the total IL-17A⁺ cells.

2.5 | Cell cultures

Human lung microvascular endothelial cells (HMVEC-L) derived from 4 different healthy donors were purchased from Lonza and grown in EGM-2MV medium (human basal endothelial cell medium [EBM-2] supplemented with 5% FBS and growth factors [GF]) (hydrocortisone, bFGF, VEGF, insulin-like growth factor, ascorbic acid and gentamicin/amphotericin-B; Lonza). Normal human bronchial epithelial cells (NHBE), bronchial fibroblasts (NHBF) and airway smooth muscle cells (ASMC) were isolated from 9 healthy human lung tissues, as described in details in the supplemental material section. The research project (Human lungs/2017-2800) was approved by an institutional review board.

Normal bronchial epithelial cells were cultured in BEGMTM Medium (human basal epithelial medium [BEBM] supplemented with GF (Bovine Pituitary Extract, hydrocortisone, human epidermal growth factor, epinephrine, transferrin, insulin, retinoic acid, triiodothyronine and gentamicin/amphotericin-B; Lonza). NHBF and ASMC were grown in Dulbecco's modified Eagle's medium (DMEM) containing streptomycin, penicillin and amphotericin-B (Anti-Anti) (Thermo Fisher Scientific) and 10% fetal bovine serum (FBS) (Thermo Fisher Scientific). All cells were grown in a humidified atmosphere of 95% air – 5% CO₂ at 37°C and passaged at 80% confluence. All cells in this study were used from passages 2 to 5, excepted NHBF that were used until passage 6. For all the experiments, hydrocortisone was excluded from the GF of HMVEC-L and NHBE media.

2.6 | Cell proliferation

Human lung microvascular endothelial cells were seeded in 24-well plate (1.5×10^4 cells/well) and allowed to reach 70% of confluence in complete medium. Cells were starved for 16 hours in EBM-2 + GF + 0.5% FBS and then incubated with IL-17A (10–100 ng/mL) or vehicle only (4 mmol L^{-1} HCl in PBS). 24 hours before the end of the experiment, cells were pulsed with 2 $\mu\text{Ci/well}$ of ³H-thymidine. DNA was isolated from the cells with a TomTek Micro96 Cell Harvester (TomTec) and then radioactivity was measured with a scintillation counter (PerkinElmer). Cells maintained in EGM-2MV were used as a positive control. For each independent experiment, each condition was performed in duplicate. For the experiments carried out in 2.5% FBS and 5% FBS (Figures S1–S4) and with conditioned medium (CM) cell proliferation was evaluated by BrdU incorporation (Roche Diagnostic) following manufacturer's instructions. For each independent experiment, each condition was performed in quadruplicate.

2.7 | In vitro tube formation

Ninety-six-well plates were coated with Matrigel™ (8.9 mg/mL; BD Biosciences), and HMVEC-L were seeded on the polymerized matrix at a density of 1.5×10^4 cells/well in EBM-2 + 0.5% FBS in the presence of IL-17A or vehicle only. bFGF (20 ng/mL) was used as a positive control.

Phase contrast pictures were acquired every 2.5 hours up to 24 hours, with bright field microscope (Evos XL Core, ThermoFisher Scientific) at 4× and 10× magnification. The analysis was performed by Angiogenesis Analyzer by ImageJ (NIH) on the images (4× magnification) acquired at 5 hours after the starting of the experiments when the maximal cell differentiation was observed. For each independent experiment, each condition was performed in triplicate.

2.8 | Cell migration

Cell migration was evaluated by scratch wound healing assay in ninety-six well-plates. Phase contrast pictures were acquired with bright field microscope (ThermoFisher Scientific) at time 0 and 24 hours after the start of the experiments. EBM-2 + GF was used as positive control.

The area of the well free of cells was quantified by ImageJ (NIH) and cell migration was determined as % of scratch area closure at 24 hours compared to 0 hours. For each independent experiment, each condition was performed in quadruplicate.

2.9 | Measurement of trans-endothelial electric resistance (TEER)

Gold electrodes were kindly donated by Applied BioPhysics (Troy). Wells were equilibrated with 10 mmol L⁻¹ L-cysteine (Sigma-Aldrich) and then coated with 0.1% gelatin (Sigma-Aldrich) or a solution of laminin and collagen IV (10 and 100 µg/mL, respectively, Sigma-Aldrich). Cells were seeded at 5×10^4 cells/well in complete medium and allowed to attach. A constant current of 1 A was applied and 4000 and 64 000 Hz were used to monitor resistance and capacitance, respectively. Once capacitance reached a value of 5–10 nF and the resistance was relatively constant (1400–2000 Ohm), cells were equilibrated in EBM-2 + GF+2.5%FBS for 4 hours and then incubated with IL-17A (100 ng/mL) or vehicle alone. Thrombin (2 U/mL; Sigma-Aldrich) was used as a positive control. For each independent experiment, each condition was performed in triplicate.

2.10 | Analysis of the production of angiogenic factors and CXCL8

Sputum was processed as described above. As far as concern cell supernatant, cells were seeded in 6 well plates, serum starved in EBM-2 + GF+0.5%FBS (HMVEC-L), BEBM+GF (NHBE) or DMEM+1%FBS (NHBE and ASMC) for 16 hours and then incubated with IL-17A (10–100 ng/mL) or vehicle. After 24 hours of incubation, the supernatants were collected, centrifuged at 3000 rpm for

10 minutes at 4°C and stored in aliquots at –80°C. Cells treated with 10 ng/mL TNF- α or 40 nmol L⁻¹ phorbol myristate acetate (PMA, an activator of protein kinase C; Sigma-Aldrich) were used as a positive control. Sputum and cell supernatant were processed for the analysis by a bead-based suspension array (Luminex xMAP Technology; Millipore) or CXCL8 ELISA (BioLegend), according to the manufacturer's protocol. The factors detectable by the array and relative range of detection are reported in the Supporting Information.

2.11 | Analysis of mRNA expression

RNA was isolated by NucleoSpin RNA kit (Macherey-Nagel) and cDNA was generated by SuperScript™ IV Reverse Transcriptase (ThermoFisher Scientific). Quantitative real-time PCR was performed using StepOne™ Real-Time PCR System (Applied Biosystems) with Power SYBR® Green PCR Master Mix (Applied Biosystems). GAPDH was used as housekeeping gene. Primer sequence is reported in the Supporting Information.

2.12 | Preparation of conditioned medium and treatment of HMVEC-L

Conditioned medium from NHBE and ASMC was collected after 24 hours of IL-17A (100 ng/mL) or vehicle stimulation, centrifuged at 1500 rpm to eliminate dead cells, pooled and stored in sterile condition at –80°C until use. HMVEC-L were incubated with CM and tested for cell proliferation by BrdU incorporation, tube formation and cell migration as previously described. The condition CM NHBE+ASMC refers to the combination of CM from NHBE and ASMC (ratio 1:1). To prevent cell death, CM was diluted with a mixture of EBM-2 + 2%FBS (ratio 4:1).

2.13 | Statistical analysis

All results were reported as means $\pm$ SEM, except as otherwise stated. Statistics were performed using Graph Pad Prism 6.0 software. The χ^2 test was used to compare subject characteristics. For the analysis of vascularity, normality of distribution was checked with Kolmogorov-Smirnov test. Statistical comparisons between groups in terms of % vascular area and number of vessel/mm² were performed with one-way analysis of variance (ANOVA) for normally distributed data and Kruskal-Wallis tests for skewed data, respectively, accompanied by post hoc tests for multiple comparisons (Bonferroni and Dunn's, respectively). To examine the associations between lung function tests, sputum cells, angiogenic factors and vascularity, non-parametric Spearman correlation analysis was performed. Analysis of TEER was performed using repeated-measures ANOVA, and post hoc analysis was performed with a Bonferroni multiple-comparison test. For remaining data, non-parametric test for paired data was used with Dunn's post hoc tests for multiple comparisons. Wilcoxon matched pairs-signed rank test was used to test the statistical difference of cells treated with positive control compared to vehicle-treated cells. A P-value of <.05 was considered statistically significant.

3 | RESULTS

3.1 | Vascularization is increased in severe asthma and positively correlates with the concentration of angiogenic factors in the sputum

Severe asthmatic had a significantly increased percentage of vascular area compared to healthy, mild and moderate asthmatics as well as COPD subjects (Figure 1A and B). The density of vessels in the submucosa was approximately 2-fold higher in severe asthmatic than in healthy subjects ($P < .05$) (Figure 1C). FEV₁ (% predicted) weakly and negatively correlates with the density of vessels in asthmatic subjects ($P = .03$, $r = -.36$; Figure 1D) but not with the percentage of vascular area ($P = .09$, $r = -.29$; Figure 1E). The analysis of angiogenic factors in the sputum revealed a positive correlation between vascularity (expressed as % of vascular area) and epithelial growth factor (EGF), G-CSF, endothelin-1, bFGF, vascular endothelial growth factor (VEGF)-D (Figure 1F). No correlations between the number of vessels/mm² and the concentration of angiogenic factors in the

sputum were found (data not shown). IL-17A was not present in detectable quantities (data not shown).

3.2 | Bronchial vascularization and asthma severity positively correlates with the number of IL-17A⁺ cells in the submucosa

The number of IL-17A⁺ cells in the submucosa correlated positively with the percentage of vascular area ($P = .01$; $r = .30$) and negatively with FEV₁ (% predicted, $P = .006$; $r = -.59$; Figure 2). No correlation was found with the vessel density in the submucosa (data not shown).

We characterized the inflammatory cells expressing IL-17A in the submucosa. Despite the fact that all the cells analysed can express IL-17A (Figure 2D), neutrophils represent the major population followed by macrophages (Figure 2E). No significance correlation has been found between the % of IL-17A⁺ cell represented by neutrophils and vascular index as well as lung function (Figure 2F; FVC [% predicted] not shown).

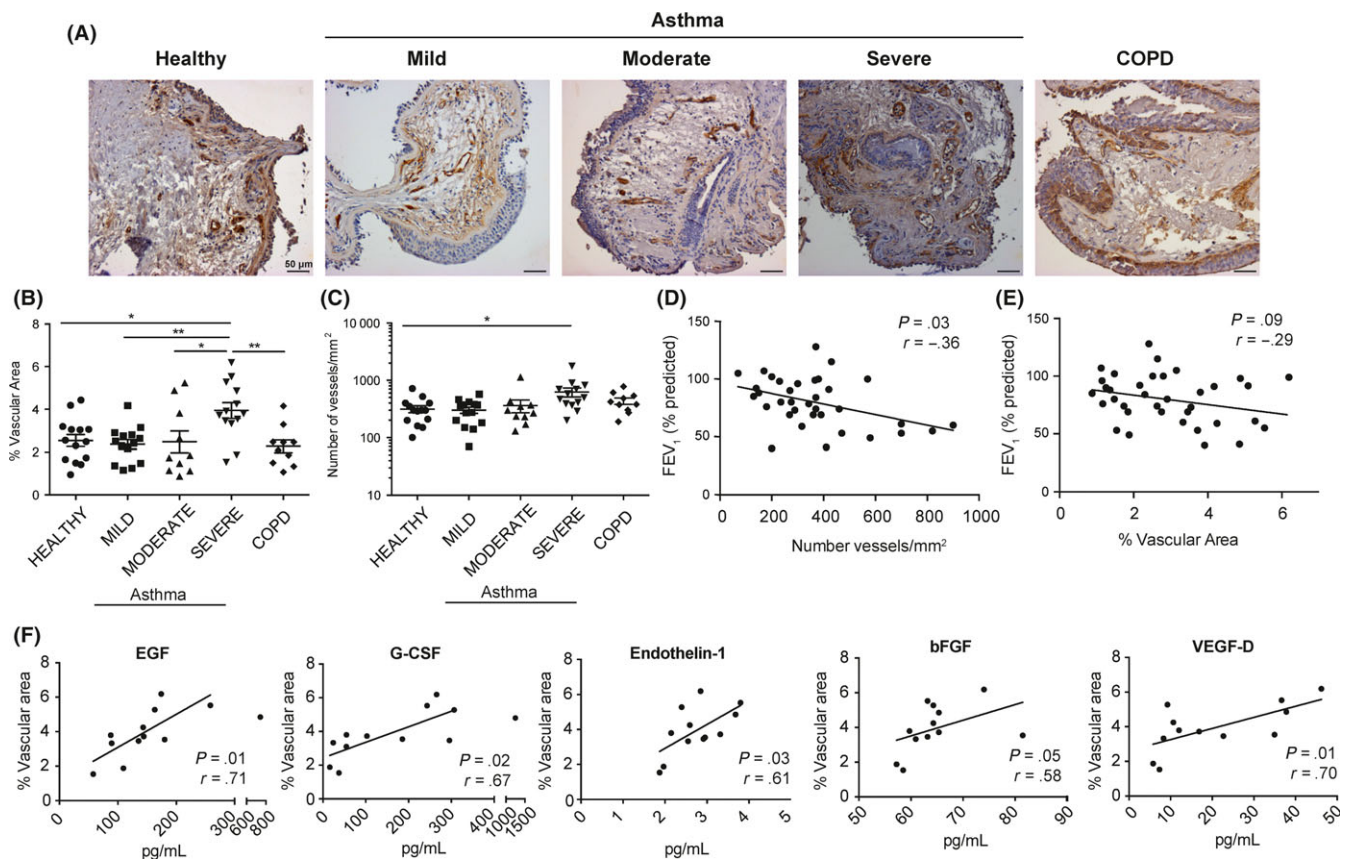


FIGURE 1 Vascularity is increased in severe asthma and positively correlates with asthma severity and the concentration of angiogenic factors in the sputum. (A) Sections derived from bronchial biopsies of healthy ($n = 14$), mild ($n = 14$), moderate ($n = 10$) and severe ($n = 13$) asthmatic, and COPD ($n = 10$) subjects were stained with anti-vWF. For each group, vascular index was expressed as the percentage of the total vascular area normalized to the subepithelial area beneath the epithelial basement membrane (excluding mucosal glands and smooth muscle bundles) (B) and as a number of vessel per mm² (C). Correlation between FEV₁ (% predicted) and percentage of vascular area (D) and number of vessels per mm² (E) in asthmatic subjects. (F) Positive correlation between vascularity expressed as % of vascular area and the concentration of EGF, G-CSF, Endothelin-1, bFGF, VEGF-D (pg/mL) in the sputum of severe asthmatic patients ($n = 12$; subject 50 not available). * $P < .05$; ** $P < .01$. COPD, chronic obstructive pulmonary disease

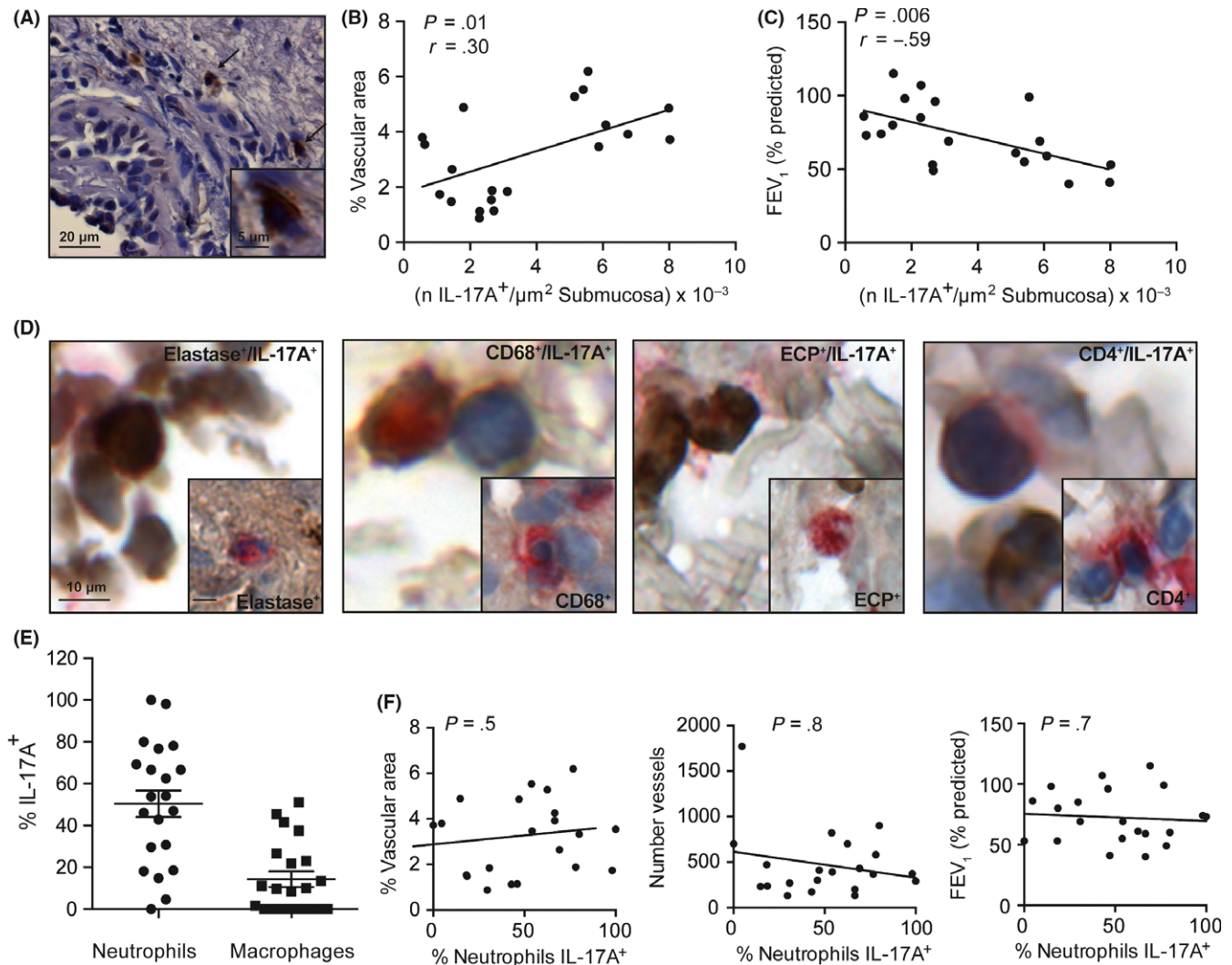


FIGURE 2 Bronchial vascularity and asthma severity positively correlate with the number of cells positive for IL-17A in the submucosa. (A) Representative bronchial biopsy stained with anti-IL17A (arrows). Inserted square represents a magnification of IL-17A⁺ cell. (B) and (C), Correlation between the number of IL-17A⁺ cells in the submucosa and the % vascular area or with the number of vessels in moderate and severe asthmatic subjects. Scale bar: 10 μ m for all pictures. (D) Representative images of cells double stained for IL-17A and marker of inflammatory cells (large square) or single stained (inserted square). (E) quantification of % of IL-17A⁺ expressed by neutrophils or macrophages. (F) correlation between the % of IL-17A⁺ neutrophils and vascular index and lung function (subjects 38 and 40 not available)

3.3 | IL-17A does not affect cell proliferation, in vitro angiogenesis, cell migration and vascular permeability of HMVEC-L

To investigate the effect of IL-17A on vascular remodelling, we used HMVEC-L, a frequently used model of lung microvasculature.^{20,21} Biocompatibility of IL-17A, vehicle, bFGF, PMA and TNF- α on HMVEC-L was demonstrated by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test (Figure S1a). IL-17A did not affect the proliferation of HMVEC-L under any of the conditions tested (Figure 3A; Figure S1b and c) and did not promote angiogenesis, as measured by in vitro tube formation (Figure 3B). Similarly, we did not find differences in cell migration (Figure 3C). Time course and dose-response have been provided as supplemental information in Figure S2.

As vascular permeability index positively correlates with asthma severity, we investigated if IL-17A perturbed the endothelial barrier integrity of the HMVEC-L monolayer. We found that IL-17A did not affect endothelial barrier when compared to vehicle up to 24 hours (Figure 3D). Similar results were observed when gelatin was replaced by a solution of collagen IV/laminin to mimic the endothelial basement membrane (Figure S1d).

3.4 | IL-17A induces the production of CXCL8 and G-CSF but not that of other angiogenic factors

Human lung microvascular endothelial cells were exposed to cytokines or vehicle alone for 24 hours and the supernatants were analysed for the expression of angiogenic factors and CXCL8. Among seventeen factors tested only CXCL8 and G-CSF were significantly

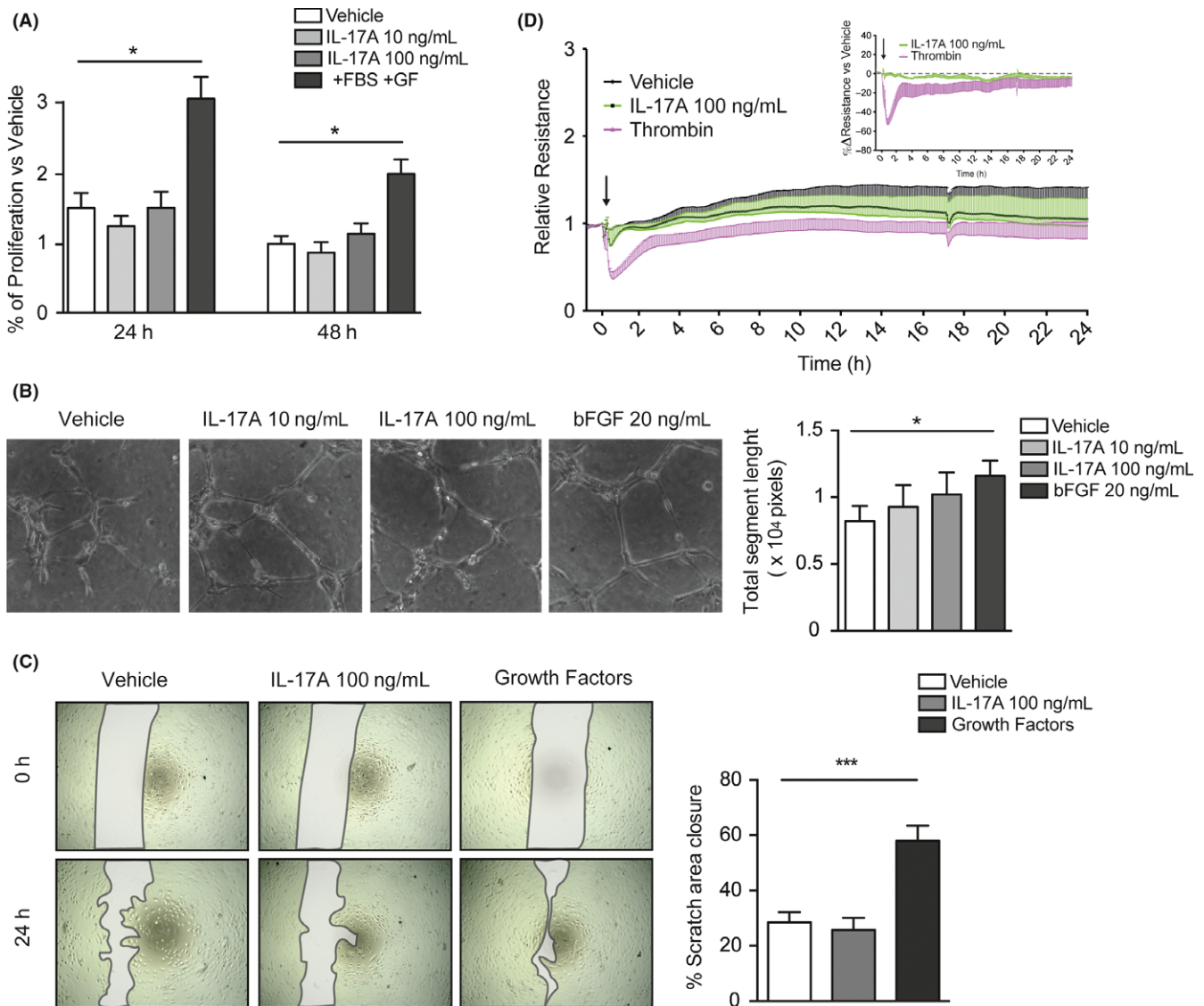


FIGURE 3 IL-17A does not directly affect proliferation, angiogenesis, cell migration and vascular permeability in HMVEC-L. (A) Proliferation analysis of HMVEC-L treated with IL-17A ($n = 4$). (B) Representative pictures of in vitro tube formation after stimulation with IL-17A, vehicle or bFGF and quantification of total network length per well ($n = 8$). (C) Representative images of scratch wound healing assay before and after 24 h of treatment with IL-17A, vehicle or EBM-2 + growth factor. Migration of cells was expressed as % of scratch area closure ($n = 4$). (D) Effects of IL-17A on endothelial barrier function of cells seeded on gelatin-coated wells. Resistance values at each time-points were normalized to the value registered at $t = 0$ h (the arrow corresponds to the addition of the stimulus) and the averaged values obtained among the 4 donors were reported in the graph. The resistance was also expressed as variation with respect to the resistance of vehicle treated cells for each donor and the averaged values were reported in the insert graph (FBS, fetal bovine serum; GF, growth factors). The graphs reported the mean + SEM. Arrow indicates the addition of the stimuli ($n = 4$) * $P < .05$, *** $P < .001$

induced by IL-17A, at the protein and mRNA levels (Figure 4A, B). Treatment with an anti-IL-17A neutralization antibody prevented these increases (data not shown). No changes in VEGF-A expression was noted after IL-17A treatment (Figure 4A, B). Further details are reported in the Table S3, in the supporting information.

3.5 | miRNA profiling is unchanged after IL-17A stimulation

Certain miRNA are established as key regulators of proliferation and activation of endothelial cells and angiogenesis.²² To further

investigate mechanisms behind the pro-angiogenic effects of IL-17A, we performed a miRNA profiling on HMVEC-L treated with IL-17A 100 ng/mL and compared results to vehicle-treated cells. However, there were no significant changes in miRNA expression in IL-17A-treated samples compared to vehicle-treated, a finding we attribute to high inter-donor variability (see Figure S3a and b). Although the analysis of miRNA did not give us any further insights into the effect of IL-17A on vascular remodelling, the data provided additional supporting information that direct effects of IL-17A on endothelial cell gene expression are weak.

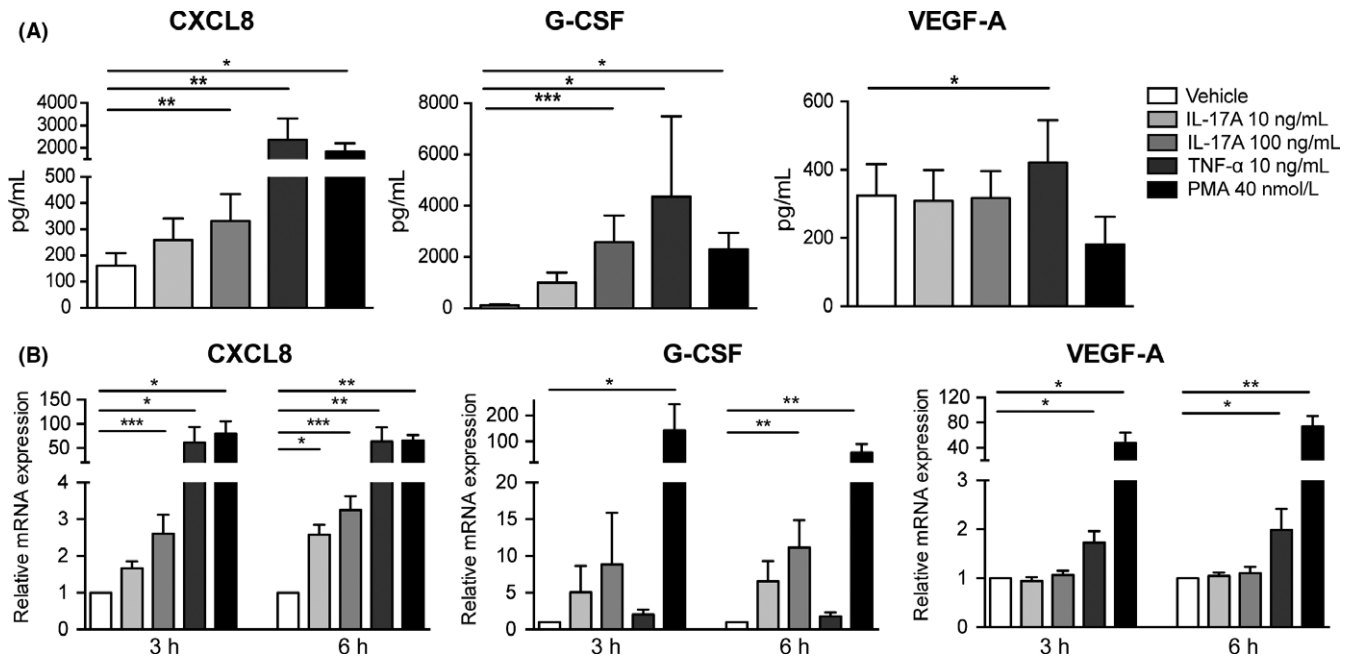


FIGURE 4 IL-17A induces the production of CXCL8 and G-CSF by regulating gene transcription. (A) Concentrations of CXCL8, G-CSF and VEGF-A in the supernatant of HMVEC-L treated for 24 h with vehicle and IL-17A ($n = 7$ or 8). (B) Analysis of mRNA level at 3 and 6 h after the application of the stimuli ($n = 8$). For both experiments, TNF- α and PMA were used as positive controls. * $P < .05$, ** $P < .01$, *** $P < .001$

3.6 | IL-17A induces the production of several angiogenic factors in NHBE and ASMC

Based on the above findings, we reasoned that IL-17A might indirectly influence endothelial function through effects on other types of structural cells. Therefore, we stimulated NHBE, NHBF and ASMC mono-cultures and analysed the expression of angiogenic factors in their supernatants, as was previously performed in HMVEC-L. IL-17A induced the secretion of CXCL8 and G-CSF in all the cell types analysed (Table 2). Moreover, in accordance with our hypothesis we found that in NHBE and ASMC cells, IL-17A stimulated the production of several pro-angiogenic factors when compared to vehicle-treated cells (Table 2).

3.7 | IL-17A indirectly promotes in vitro angiogenesis and cell migration in HMVEC-L

Human lung microvascular endothelial cells were exposed to conditioned medium derived from NHBE and ASMC treated with IL-17A or vehicle, and consequently, tested for proliferation, tube formation and cell migration.

Conditioned medium derived from IL-17A treated cells did not affect HMVEC-L proliferation (Figure 5A) but augmented the capacity to form an endothelial network and to migrate when compared to CM derived from vehicle-treated cells (Figure 5B, C). Despite a similar trend in all conditions, only CM from NHBE treated with IL-17A resulted in, significantly, more angiogenesis compared to CM from NHBE-vehicle treated (Figure 5B). Moreover, the combination of CM derived from NHBE and ASMC IL-17A

treated cell increased endothelial cell migration compared to vehicle alone (Figure 5C).

4 | DISCUSSION

The bronchial vasculature maintains airway temperature, hydration and provides oxygen and nutrients to the bronchial tissues.²³ However, once the airways are cooled by inhalation of cold air the subsequent re-warming in the asthmatic occurs more rapidly reflecting increased blood flow and is a postulated mechanism of exercise-induced asthma.^{24,25} Bronchial vascular remodelling and the bronchial vascular blood volume may contribute to airway narrowing through mucosal congestion, airway oedema, inflammation and wall thickening.²⁶ Changes to the architecture of wall vessels, angiogenesis, increased vascular permeability and endothelial cell activation are the main processes that characterize vascular remodelling. Among these alterations, angiogenesis, the process by which new capillaries are formed from pre-existing vessels, has attracted particular interest in asthma. In fact, the degree of bronchial vascularity correlates with disease severity and airway hyperresponsiveness.²⁷ In the current study, we found that both the percentage of vascular area and number of vessels are significantly increased in biopsies derived from neutrophilic severe asthmatics compared to those from mild and moderate asthmatics and healthy subjects. In addition, despite the weakness of the correlation, we confirmed that disease severity positively correlates with vessel density in the submucosa of asthmatic patients. In recent years, much literature has been dedicated to vascular remodelling in COPD especially with regard to the more severe

TABLE 2 Concentration of angiogenic factors (pg/mL) detected in the supernatant of NHBE, ASMC and NHBF after 24 h of incubation with IL-17A with respect to vehicle-treated cells

	Target	Vehicle	IL-17A 10 ng/mL	IL-17A 100 ng/mL	TNF- α 10 ng/mL	PMA 40 nmol L ⁻¹
NHBE	EGF	7.1 $\pm$ 1.0	7.3 $\pm$ 0.8	7.1 $\pm$ 0.8	9.1 $\pm$ 1.8	28.4 $\pm$ 6.6**
	Angiopoietin-2	19.3 $\pm$ 0.8	20.2 $\pm$ 0.9	20.4 $\pm$ 1.2	20.1 $\pm$ 0.6	22.9 $\pm$ 1.5
	CXCL8	256.6 $\pm$ 120.7	749.2 $\pm$ 328.6	585.9 $\pm$ 166.5***	1642.0 $\pm$ 471.8**	3099.6 $\pm$ 600.3**
	G-CSF	423.3 $\pm$ 123.6	7108.8 $\pm$ 2053*	8791.4 $\pm$ 1727.9***	2096.3 $\pm$ 625.8	6864.1 $\pm$ 1744.6**
	Endothelin-1	11.6 $\pm$ 2.4	22.5 $\pm$ 3.7	30.7 $\pm$ 6.1****	48.3 $\pm$ 15.5	38.4 $\pm$ 6.4**
	FGF-1	14.3 $\pm$ 1.5	15.4 $\pm$ 1.6	15.3 $\pm$ 1.6	17.7 $\pm$ 2.4	18.3 $\pm$ 1.6**
	Follistatin	4129.9 $\pm$ 1053.4	5886.7 $\pm$ 1820.1	6119.6 $\pm$ 1318.1*	3058.2 $\pm$ 1860.4	589.1 $\pm$ 194.5**
	HGF	29.3 $\pm$ 1.7	41.0 $\pm$ 13.1	33.7 $\pm$ 6.2	31.6 $\pm$ 5.9	35.6 $\pm$ 5.9
	HB-EGF	5.5 $\pm$ 0.5	6.1 $\pm$ 0.6	6.8 $\pm$ 0.7***	8.1 $\pm$ 1.2	134.6 $\pm$ 15.9**
	PLGF	508.1 $\pm$ 60.2	476.2 $\pm$ 50.0	497.2 $\pm$ 55.8	624.6 $\pm$ 116.0	704.8 $\pm$ 69.6*
	VEGF-A	4340 $\pm$ 848.8	4363 $\pm$ 855.9	5396.4 $\pm$ 1329.7*	5868.7 $\pm$ 1377.7	9746.0 $\pm$ 2459.3**
	VEGF-C	540.6 $\pm$ 87.6	516.0 $\pm$ 85.9	591.4 $\pm$ 101.4	821.5 $\pm$ 205.6*	405.1 $\pm$ 62.5
	VEGF-D	11.8 $\pm$ 1.6	14.9 $\pm$ 1.4*	15.7 $\pm$ 1.4***	12.1 $\pm$ 2.3	15.7 $\pm$ 2.0**
	FGF-2	191.5 $\pm$ 15.3	192.4 $\pm$ 14.3	185.1 $\pm$ 16.5	198.3 $\pm$ 22.0	338.3 $\pm$ 21.2**
NHBF	Angiopoietin-2	56.2 $\pm$ 8.3	43.2 $\pm$ 6.3	43.2 $\pm$ 6.3	32.5 $\pm$ 3.3*	22.6 $\pm$ 1.5**
	CXCL8	331.9 $\pm$ 91.8	447.4 $\pm$ 128.8*	534.7 $\pm$ 137.6***	5996.4 $\pm$ 2022**	2891.8 $\pm$ 1160.7**
	G-CSF	349.8 $\pm$ 107.2	504.2 $\pm$ 159.9*	594.1 $\pm$ 170.3**	8776.5 $\pm$ 1607.4*	4647.2 $\pm$ 1252.1*
	Follistatin	9418.3 $\pm$ 1920.5	9189.0 $\pm$ 1467.3	10372.6 $\pm$ 2476.9	18686.0 $\pm$ 2641.0*	612.4 $\pm$ 267.1**
	HGF	4438.3 $\pm$ 497.2	4731.4 $\pm$ 536.9	5141.0 $\pm$ 692.2	4876.1 $\pm$ 783.1	15914.6 $\pm$ 1946.1**
	VEGF-A	1193.6 $\pm$ 139.7	1242.3 $\pm$ 151.1	1309.8 $\pm$ 191.1	1265.5 $\pm$ 176.4	5244.0 $\pm$ 790.9**
	VEGF-C	650.8 $\pm$ 91.9	644.0 $\pm$ 83.2	619.9 $\pm$ 77.4	1575.4 $\pm$ 296.2**	1108.1 $\pm$ 156.8**
	FGF-2	105.1 $\pm$ 16.6	105.8 $\pm$ 16.8	107.4 $\pm$ 15.2	112.7 $\pm$ 17.7	121.4 $\pm$ 8.4
ASMC	Angiopoietin-2	19.5 $\pm$ 1.3	20.9 $\pm$ 2.1	21.3 $\pm$ 1.8	22.5 $\pm$ 1.6*	20.9 $\pm$ 1.3
	CXCL8	627.8 $\pm$ 233.6	851.3 $\pm$ 293.0	1051.5 $\pm$ 365.5****	4035.1 $\pm$ 834.6*	1933.8 $\pm$ 350.0*
	G-CSF	160.0 $\pm$ 31.0	402.3 $\pm$ 110.1	465.8 $\pm$ 116.5***	3755.3 $\pm$ 870.4**	2723.4 $\pm$ 468.1**
	Follistatin	4373.3 $\pm$ 450.4	5184.4 $\pm$ 344.9	5597.9 $\pm$ 397.4**	15518.0 $\pm$ 2653.7*	1500.5 $\pm$ 403.3**
	HGF	4594.1 $\pm$ 1329.8	5614.0 $\pm$ 1782.1	5737.7 $\pm$ 1759.3	6916.4 $\pm$ 2001.3*	18484.4 $\pm$ 4456.1**
	PLGF	36.2 $\pm$ 9.1	51.7 $\pm$ 14.0*	55.8 $\pm$ 14.5**	39.3 $\pm$ 8.6	19.7 $\pm$ 3.7
	VEGF-A	440.4 $\pm$ 68.1	519.2 $\pm$ 87.12	559.2 $\pm$ 94.3**	583.1 $\pm$ 88.8*	6417.1 $\pm$ 1354.3**
	VEGF-C	297.2 $\pm$ 42.2	343.1 $\pm$ 48.2**	351.1 $\pm$ 50.8**	600.1 $\pm$ 85.6**	736.9 $\pm$ 108.3**
	FGF-2	106.9 $\pm$ 15.0	109.1 $\pm$ 14.7	105.5 $\pm$ 16.0	112.8 $\pm$ 13.7	114.4 $\pm$ 11.7

ASMC, airway smooth muscle cells; NHBE, normal bronchial epithelial cells; NHBF, bronchial fibroblasts; EGF, endothelial growth factor.

Factors that were undetectable in both vehicle and IL-17A treated cells were omitted. Data are presented as means $\pm$ SEM (n = 6-9 for all type of cells), *P < .05, **P < .01, ***P < .001, ****P < .0001.

phenotypes (GOLD III and IV) and to the asthma-COPD overlap syndrome (ACOS). However, the data about changes in vascularization are more consistent for asthmatic subjects than for COPD where vasodilation rather than angiogenesis has been described as the predominant finding.² We demonstrated that moderate and severe COPD subjects have a lesser degree of vascularization compared to severe asthmatics and showed no difference compared to healthy and milder asthmatic subjects. In support of this, Tanaka et al performed a morphometric analysis of high-magnification bronchovideoscope of subepithelial vessels in the lower trachea and found that both vessel area density and vessel length density were significantly increased in subjects with asthma compared with control subjects

but not in patients with COPD.²⁸ The evidence that such differences exist between asthmatic phenotypes as well as COPD is crucial to better understanding asthma and to develop new therapies specific for each disease.

The reason for increased vascularity in severe asthmatics may be an excess production of pro-angiogenic factors. VEGF-A is the best-known growth factor that stimulates endothelial cell survival, blood vessel formation and vascular permeability. Interestingly, VEGF as well as other pro-angiogenic factors are increased in serum, BAL and sputum from asthmatics compared to healthy subjects.^{6,29} Of the 17 angiogenic factors that we analysed in the sputum of severe asthmatic subjects, the concentrations of EGF, G-CSF, endothelin-1,

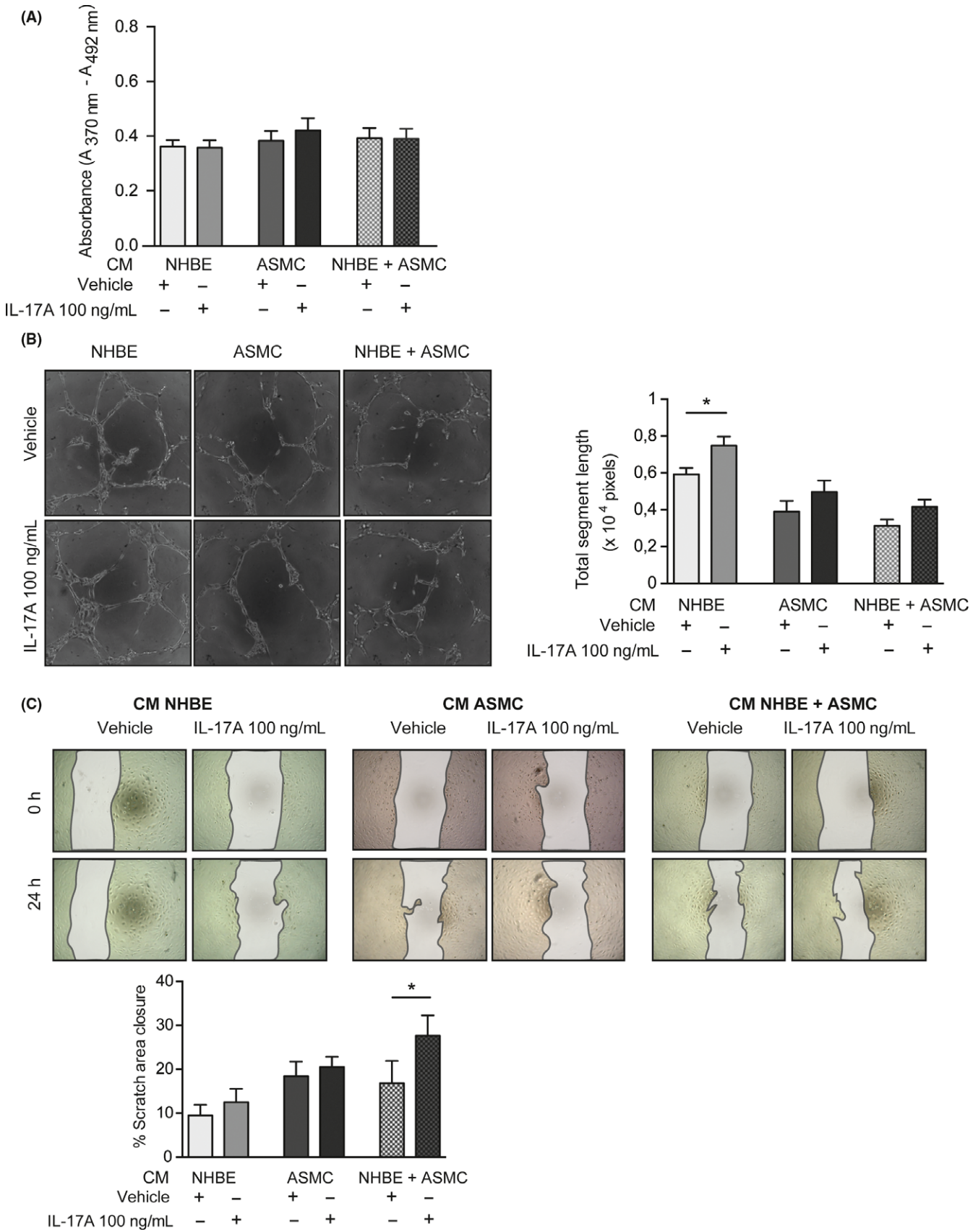


FIGURE 5 Conditioned media derived from NHBE and ASMC treated with IL-17A promotes tube formation and cell migration of HMVEC-L. A, Proliferation analysis of HMVEC-L treated with conditioned medium from NHBE and ASMC treated with IL-17A or vehicle ($n = 3$). B, Representative pictures of in vitro tube formation after stimulation with conditioned medium derived from NHBE and ASMC treated with IL-17A or vehicle. The results of the analysis of total network length per well are reported in the graph ($n = 3$). C, Representative images of scratch wound healing assay before and after 24 h of treatment with conditioned medium and quantification ($n = 3$). * $P < .05$

bFGF and VEGF-D positively correlated with the percentage of vascular area assessed in the bronchial biopsies. Besides pro-angiogenic activity, these factors are important players in airway remodelling. Indeed, EGF stimulates the proliferation of bronchial epithelial and airway smooth muscle cells³⁰; G-CSF promotes survival, proliferation and differentiation of precursors and mature neutrophils³¹; endothelin-1 is a potent bronchoconstrictor³²; and VEGF-D is involved in lymphangiogenesis.³³ Taken together these results demonstrate that vascular remodelling is sustained by a high level of many factors that support the ongoing process. Also, we cannot exclude the possibility that inhaled corticosteroid treatment may have affected the production of angiogenic factors in asthmatic subjects as well as the degree of bronchial vascularity³⁴⁻³⁶. Perhaps for this reason also the correlation of vascularity and the concentration of angiogenic factors is limited to a select few of them.

Despite the fact that many cytokines/growth factors are implicated in the development of airway remodelling, their role in vascular remodelling remains unclear. Therefore, we focused our attention to IL-17A, a pro-inflammatory cytokine elevated in severe compared to milder asthmatic phenotype,^{9,19} and one that augments angiogenesis in chronic inflammatory diseases such as rheumatoid arthritis, psoriasis and multiple sclerosis.³⁷ Despite a pro-angiogenic role of IL-17A,^{15,16} contradictory results have been described in cancer, where IL-17A inhibits tumour development and neovascularization.³⁸ In severe asthma it is still not clear whether IL-17 can promote angiogenesis by acting directly on endothelial cells or indirectly by inducing pro-angiogenic factors from cells present in the surrounding bronchial compartment. To elucidate the plausibility of a role for IL-17A in the increased vascularity, we first analysed the number of IL-17A-positive cells in the submucosa of moderate and severe asthmatic patients. As we hypothesized, we found that both bronchial vascular area and asthma severity positively correlates with the number of IL-17A⁺ cells thus suggesting its possible involvement in vascular remodelling in asthma. We also showed that neutrophils represent the major part of IL-17A⁺ cells in the submucosa. Our in vitro analysis revealed that IL-17A does not induce directly either proliferation of lung microvascular endothelial cells, in agreement with a previous report,³⁹ or in vitro tube formation and cell migration. In addition, IL-17A also did not alter vascular integrity in lung HMVEC and failed to increase the secretion of angiogenic factors. In murine pulmonary microvascular endothelial cells, IL-17A enhances angiogenesis by promoting the production of the classic angiogenic factor VEGF.¹⁴ We found however that CXCL8 and G-CSF were significantly increased by IL-17A, as previously reported by others.^{20,40} Interestingly, in addition to its primary role in the recruitment and survival of neutrophils to the lungs,⁴¹ both CXCL8 and G-CSF have been established as a potent angiogenic factor.^{42,43} MicroRNAs play an integral role in the control

of endothelial function²² and recent evidence demonstrated that they are involved in the cellular response to pro-inflammatory stimuli.⁴⁴ However we did not find a significant change in any miRNA examined after IL-17A stimulation.

When considered as a whole, our data suggest that the human lung endothelium is less susceptible to the action of IL-17A compared to endothelial cells from other organs/species. Consistently, Pickens et al have shown that IL-17A receptors are less expressed in lung HMVEC compared to skin HMVEC and HUVEC, suggesting that the latter 2 endothelial cells are more likely to respond to IL-17A in terms of tube formation and cell migration.^{15,16} Thus, inherent differences in IL-17A receptors between lung HMVEC and those from other organs may account for the observed lesser effect of IL-17A in our study. Although HMVEC-L may be the best option to serve as in vitro model of lung microvascular endothelial cells, most of the cells originate from capillaries of the pulmonary circulation whereas vascular remodelling in asthma involves mainly the bronchial circulation and have distinct phenotypic differences that may result in different biological properties.⁴⁵ Another limitation of our study is the high inter-subject variability among the donors, leaving us unable to exclude the possibility that differences may become evident with much larger sample sizes. We cannot also exclude the possibility that the expression of the IL-17A receptor may be higher in asthmatic than in non-asthmatic subjects making them more responsive to IL-17A.

TNF- α has been described to potentiate IL-17A and to promote the release of pro-inflammatory and pro-angiogenic factors in vitro.^{46,47} Therefore we investigated if proliferation, in vitro angiogenesis and cell migration would have been increased by stimulating HMVEC-L with IL-17A and TNF- α . Unfortunately, we did not find any synergistic effects between them (Figure S4).

Because our results did not support a role for direct effects of IL-17A on endothelial cells as a causative mechanism for the increase vascularity in severe asthma, we next sought to evaluate whether there were indirect actions of IL-17A via other lung structural cells. NHBE, NHBF and ASMC are a source of numerous pro-angiogenic factors that act in a paracrine fashion on surrounding endothelial cells. However, no studies have investigated their role in vascular remodelling induced by IL-17A. In our study, we found that in response to IL-17A, bronchial epithelial cells and airway smooth muscle cells secrete a number of angiogenic factors, such as the member of VEGF family, endothelin-1, follistatin, HB-EGF, PLGF, CXCL-8 and G-CSF (Table 2). The pro-angiogenic role of each of these factors has been widely demonstrated in vitro as well as in vivo and discussed in literature.⁴⁸⁻⁵⁰ Interestingly, some of these are the same factors that we found in the sputum that correlated with the vascular index in severe asthma.

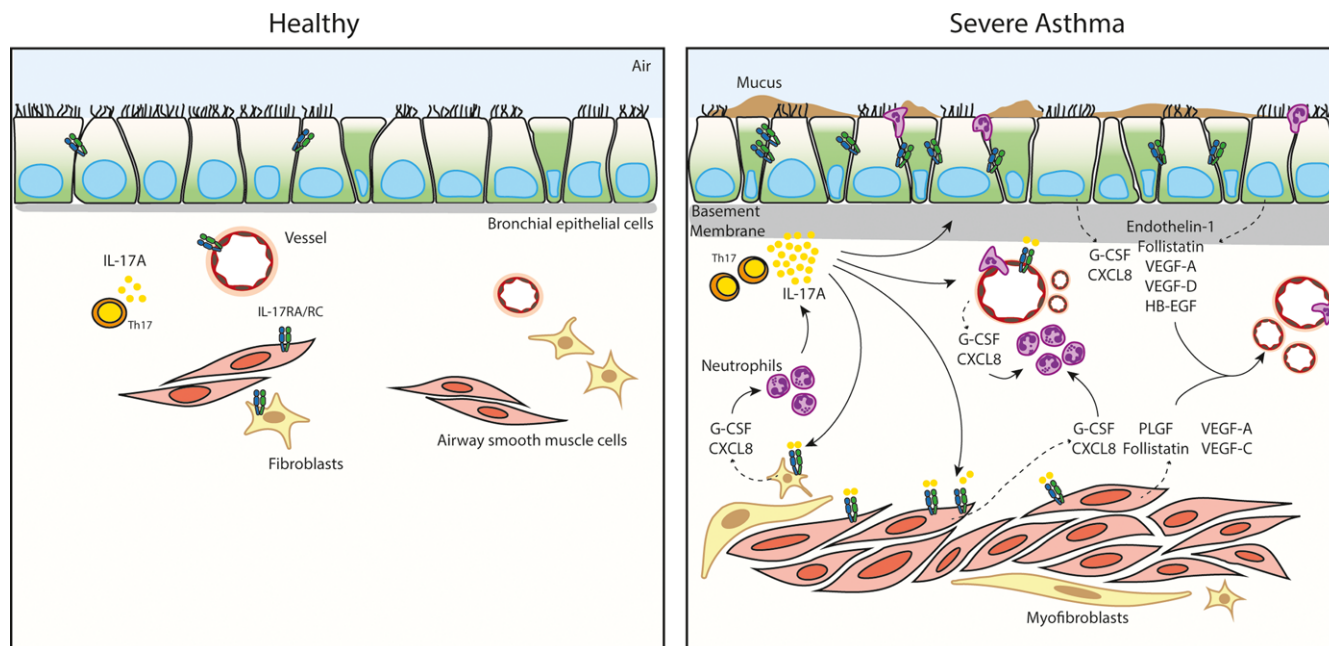


FIGURE 6 IL-17A and severe asthma. IL-17A is increased in the airways of severe asthmatic compared to healthy subjects. IL-17A participates in airway remodelling by promoting mucus secretion from airway epithelial cells, differentiation to myofibroblasts, increasing migration and contraction of airway smooth muscle cells as well as the production of interleukin-8 (CXCL8) and colony stimulatory factors (G-CSF). In addition, IL-17A stimulates the production of angiogenic factors from bronchial epithelial cells and airway smooth muscle cells thus participating in changes of submucosa vascularization

To assess whether the increased production of angiogenic factors induced by IL-17A might affect endothelial cell function, HMVEC-L were incubated with conditioned medium derived from cells treated with IL-17A. Interestingly, we found that both in vitro tube formation and endothelial cell migration were augmented by conditioned medium of cells treated with IL-17A. These data confirm the ability of IL-17A to affect angiogenesis and migration by acting on other structural cells.

In summary, we demonstrated that increased bronchial vascularity and asthma severity positively correlate with the number of IL-17A⁺ cells. We also show for the first time that any possible effects of IL-17A on vascular remodelling in the lung are almost certainly indirect, affecting endothelial function by stimulating the production of angiogenic factors from bronchial epithelial and airway smooth muscle cells (Figure 6). Targeting the effects of IL-17A on the epithelium and smooth muscle cells might prevent bronchial vascular remodelling with beneficial clinical outcomes for asthmatic patients. In vivo, the interaction between endothelial cells and other structural cells, as well as inflammatory cells and the synergism of IL-17A with other pro-inflammatory molecules may ultimately determine the pathogenic consequences of IL-17A expression in severe asthma. In this scenario it is surprising that brodalumab, an anti-IL-17R α monoclonal antibody, did not produce a treatment effect in subjects with asthma.⁵¹ However there is a need for further investigation of appropriately phenotyped subjects before excluding a utility for IL-17A in asthma. Further efforts are necessary to better elucidate the role of IL-17A on vascular remodelling and its association with airway remodelling.

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CONFLICT OF INTEREST

The authors wish to confirm that there are no known conflict of interests associated with this publication, and there has been no significant financial support for this work that could have influenced its outcome.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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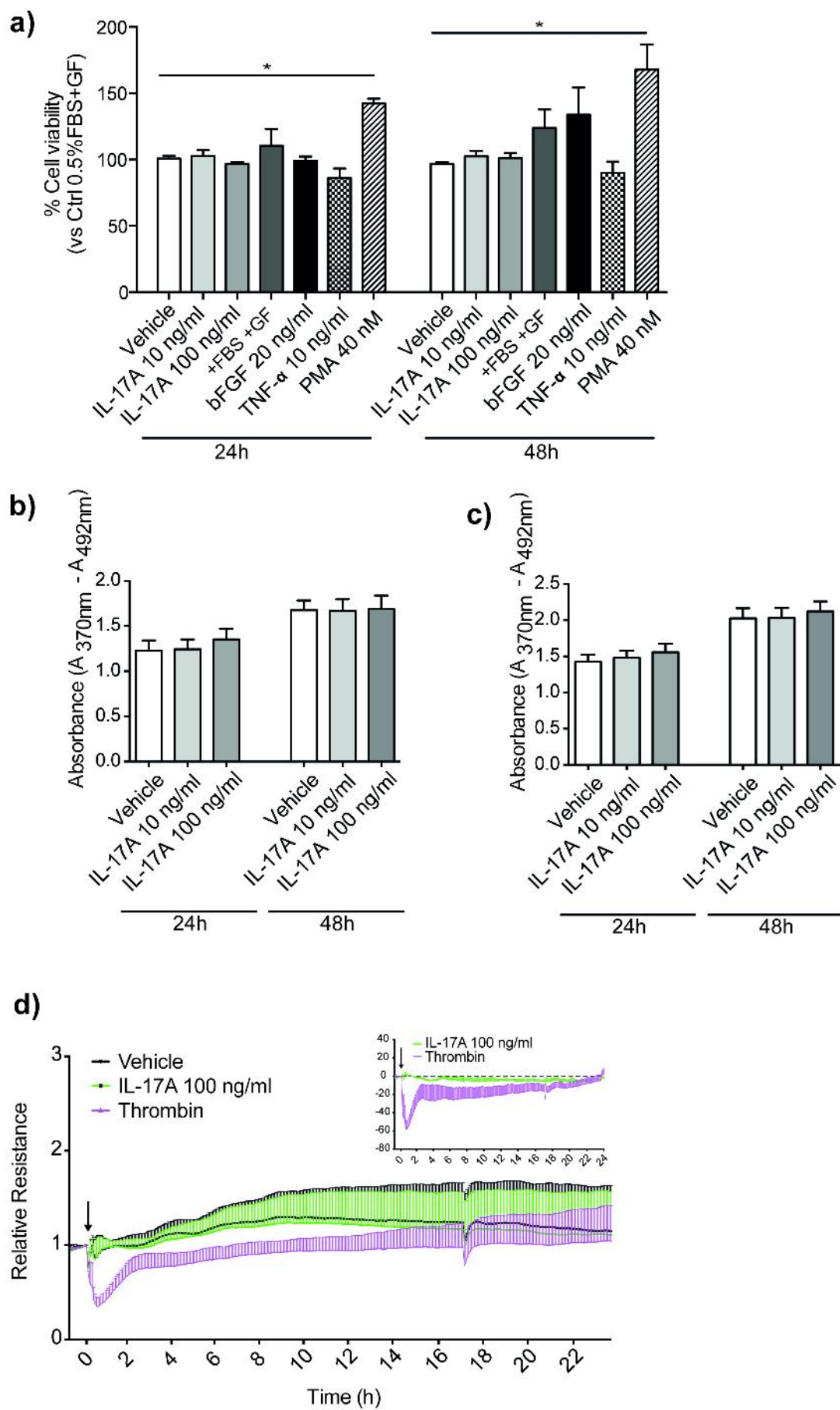


FIGURE S1

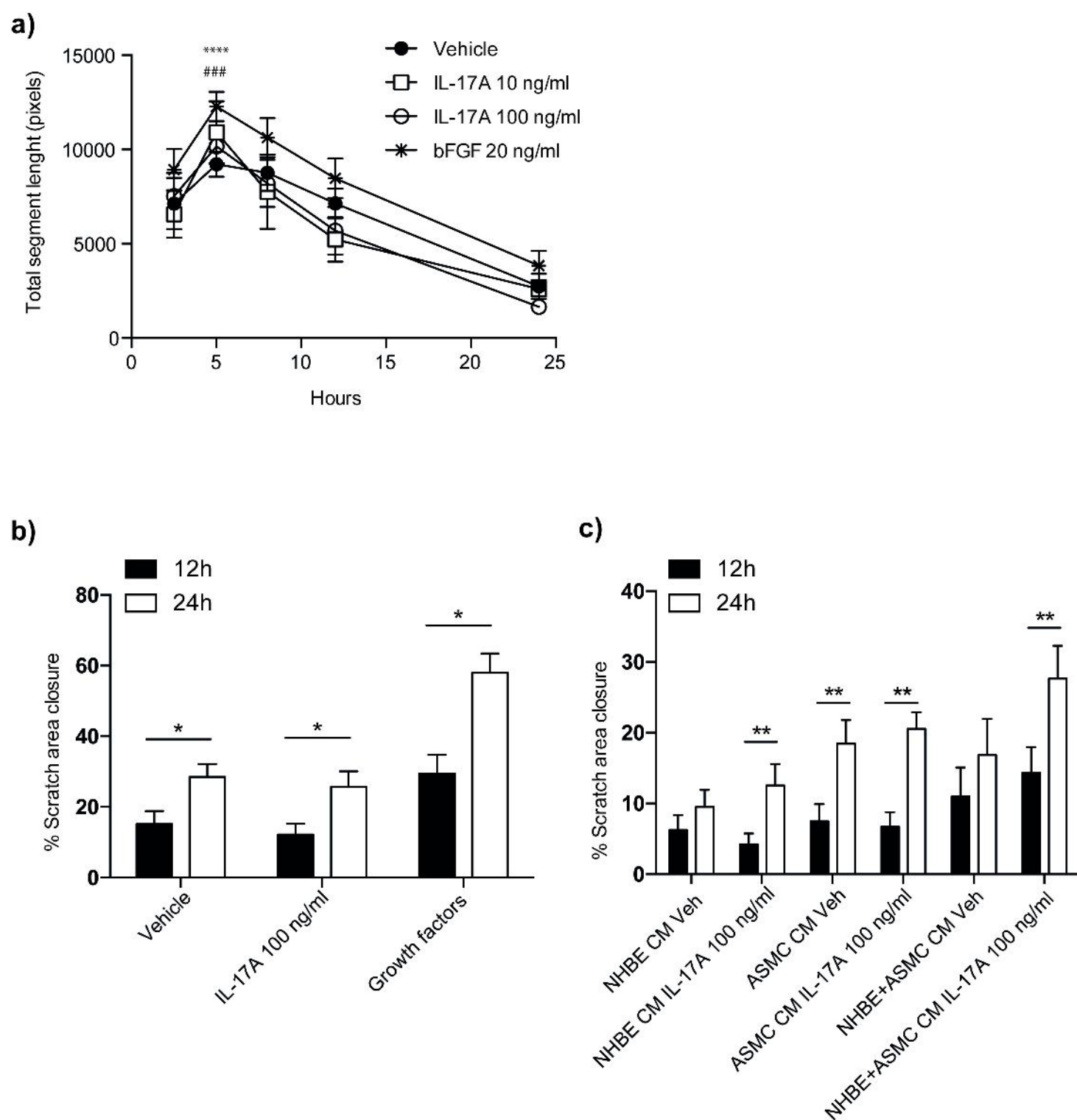


FIGURE S2

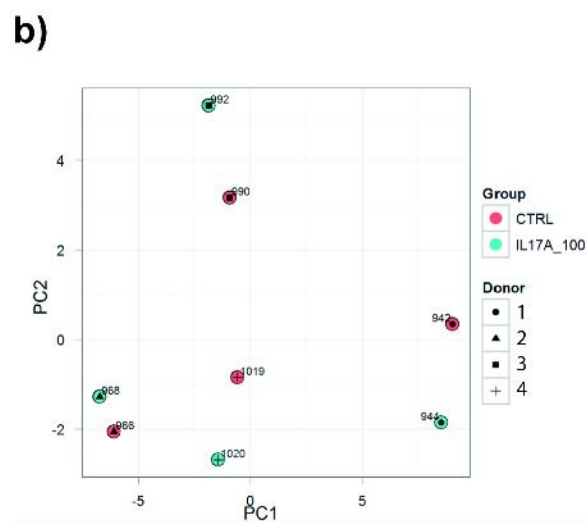
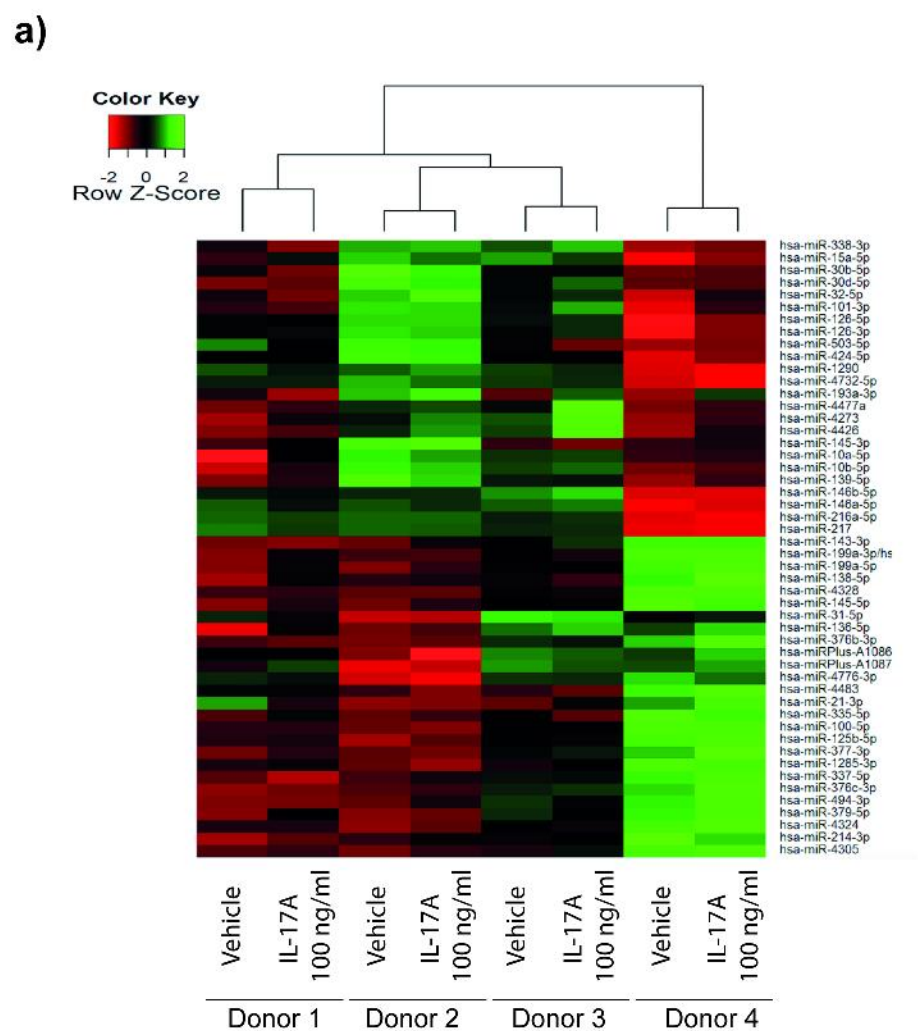


FIGURE S3

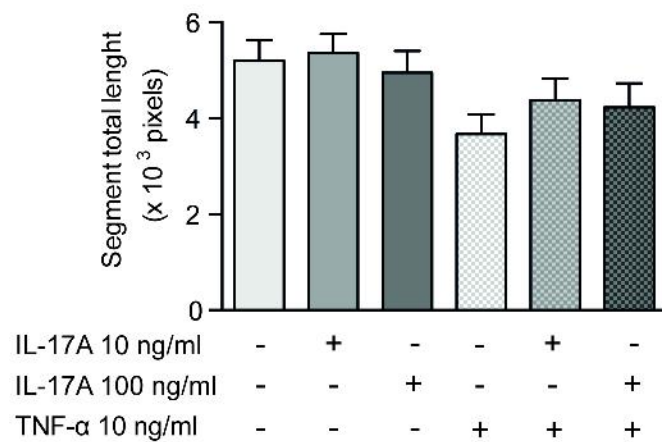
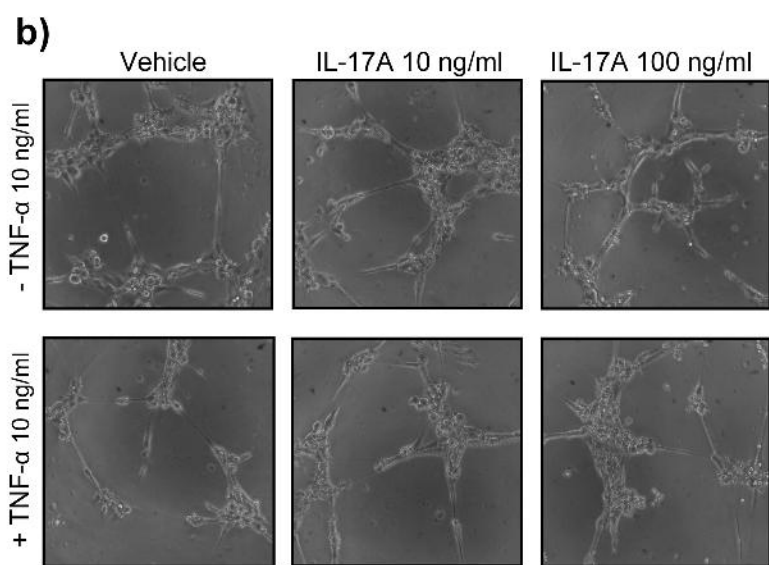
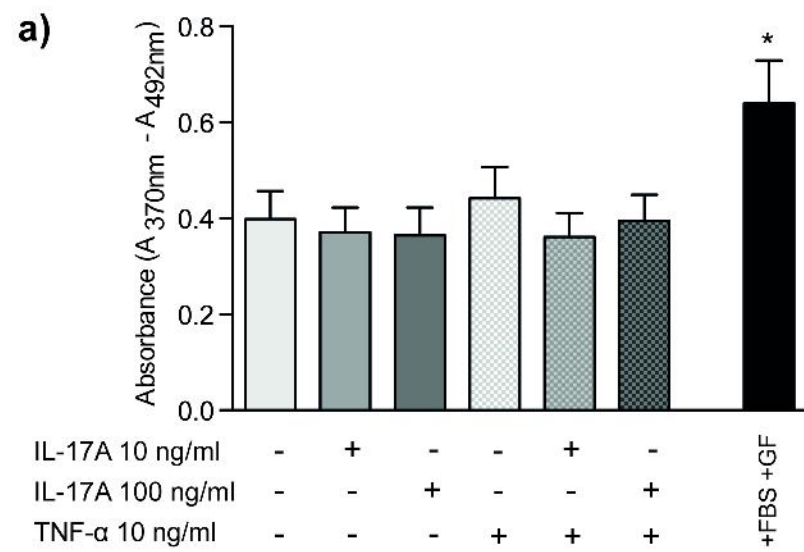


FIGURE S4

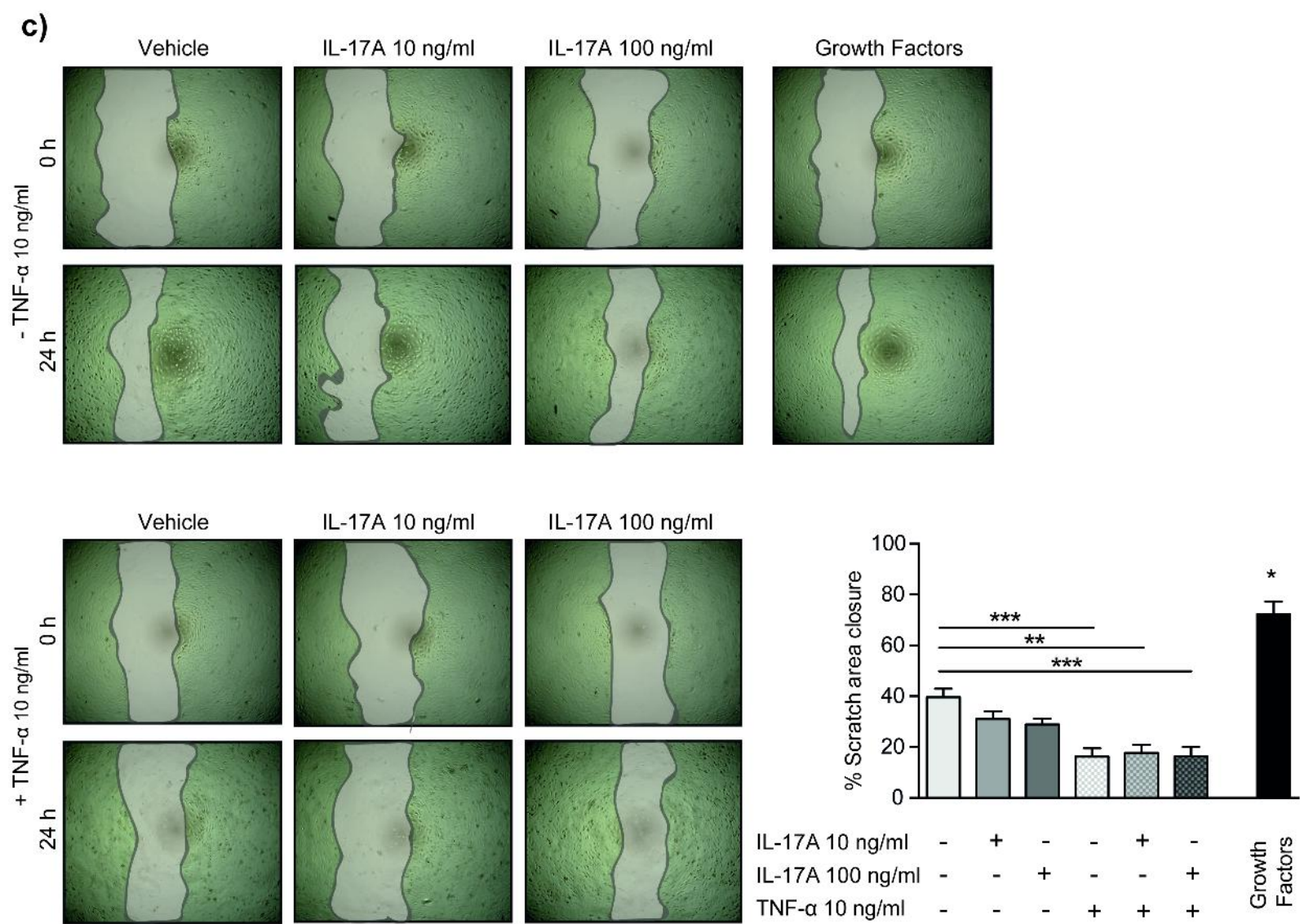


FIGURE S4

Table ST1. Detailed informations for the subject included in the study.

S, subject; A, age; G, gender; C, classification; AT, atopy; BMI, Body Mass Index; SH, smoking history; F, female; M, male; H, healthy; MildA, mild asthmatic; ModA, moderate asthmatic; SevA, severe asthmatic; COPD, chronic Obstructive Pulmonary disease Gold III/IV; NS, non smoker; S, smoker; PS, passive smoker; ES, ex smoker; N/A, not available; PRN, as needed; qd, one per day; bid, two per day; qid, four per day; qhs, every night at bedtime.

S	A	G	C	AT	BMI	SH	FEV ₁ (L)	FEV ₁ (% predicted)	FVC (L)	FVC (% predicted)	Medication
1	23	F	H	YES	20.8	NS	3.41	116	3.85	115	NONE
2	40	M	H	NO	N/A	S	4.98	123	4.98	123	NONE
3	23	F	H	YES	N/A	NS	3.12	103	3.59	106	NONE
4	45	M	H	N/A	36.4	NS	4.7	125	5.55	122	NONE
5			H			N/A					
6	29	M	H	YES	N/A	PS	5.68	125	6.82	123	NONE
7	21	F	H	YES	N/A	NS	4.74	122	5.54	121	NONE
8	20	F	H	NO	53	NS	2.93	100	3.78	97	ANOVULANT
9	59	M	H	YES	N/A	NS	3.87	105	4.67	100	NONE
10	60	F	H	NO	N/A	NS	2.38	98	2.95	97	NONE
11	30	F	H	YES	22	NS	3.83	102	5.26	117	NONE
12	28	F	H	NO	N/A	NS	3.99	120	2.02	130	NONE
13	58	M	H	NO	N/A	EX S	4.51	120	5.65	119	NONE
14	53	M	H	NO	N/A	EX S	4.22	108	5.06	103	NONE
15	22	M	MildA	YES	24	NS	3.73	80	5.22	93	Salbutamol PRN die
16	30	F	MildA	YES	N/A	NS	3.18	90	3.44	104	Terbutaline PRN die/ DEPO PROVERA 1 Injection pe rmonth/ RITALIN
17	54	M	MildA	YES	N/A	EX S	5.07	128	6.63	132	Salbutamol PRN die
18	43	F	MildA	YES	24	NS	2.23	91	3.13	110	Budesonide 400 µg, die/BEROTEC PRN
19	27	F	MildA	NO	20.9	NS	3.56	102	4.39	108	NONE
20	29	M	MildA	YES	N/A	NS	4.23	92	5.4	97	Salbutamol PRN die
21	32	M	MildA	YES	23	NS	4.05	100	4.05	84	Salbutamol PRN die
22	36	M	MildA	YES	N/A	NS	3.18	74	4.48	86	Salbutamol PRN, die/Fluticasone HFA 250 µg die
23	23	F	MildA	YES	19.4	NS	3.35	100	3.86	101	NONE
24	25	M	MildA	YES	28.8	NS	4.03	84	5.08	88	Salbutamol PRN die
25	41	F	MildA	NO	N/A	NS	2.19	88	2.89	98	Salbutamol 2 bid, die/Fluticasone HFA quid,die
26	27	F	MildA	NO	38	NS	2.7	76	3.52	85	Salbutamol PRN die
27	35	F	MildA	YES	22	NS	3.63	105	4.34	105	Budesonide 400µg die/Venlafaxine/Quetiapine
28	41	M	MildA	YES	26	EX S	2.92	69	4.55	86	Salbutamol PRN die
29	38	M	ModA	YES	29	NS	3.32	80	4.56	91	Salbutamol 100 µg die/Fluticasone/Salmeterol 2 puffs/day, 250 µg/puff
30	26	F	ModA	YES	21.2	NS	3.11	96	3.65	99	Salbutamol PRN, die/Fluticasone HFA 500ug die
31	62	F	ModA	YES	24	NS	2.46	98	3.23	100	Terbutaline 0.5 µg die/Budesonide/Formoterol 2 puffs/day, 200 µg/puff/NASAL STEROIDS 50 µg hs
32	30	F	ModA	YES	36	NS	3.18	74	4.48	86	Salbutamol 100 µg die/Budesonide/Formoterol 4 puffs/day, 200 µg/puff

33	38	M	ModA	YES	29	NS	2.99	73	4	80	Salbutamol 100 µg die/Fluticasone/Salmeterol 2 puffs/day, 250 µg/puff
34	31	F	ModA	YES	24.6	NS	2.65	85	3.49	97	Budesonide 400 µg die/Terbutaline PRN die
35	65	M	ModA	YES	29	EX S	2.21	69	3.93	96	Fluticasone HFA 500 µg die/Salbutamol PRN die
36	46	F	ModA	YES	N/A	NS	3.38	107	3.92	100	Salbutamol 100ug die/Fluticasone/Salmeterol 2 puffs/day, 125ug/puff/Montelukast 10mg die
37	40	F	ModA	YES	N/A	EX S	2.9	97	3.67	103	Fluticasone HFA 2 puffs/day, 125µg/puff/Salbutamol 100 µg die
38	44	F	ModA	NO	N/A	N/A	3.9	115	3.63	115	N/A
39	54	F	SevA	NO	N/A	EX S	1.41	53	2.39	73	QVAR 400 µg die/UNIPHYL 400 µg die/Salbutamol PRN die/Fluticasone/Salmeterol 4 puffs/day, 250 µg/puff/Montelukast 10 mg die/Esomeprazole/Analaprin/NOVOHYDROZID E/CALCIUM/FeSO4
40	64	F	SevA	YES	25	EX S	1.41	53	2.39	73	Salbutamol 100 µg die/ ASA/CALCIUM/PANTOPRAZOLE/Fluticasone/Salmeterol Diskus 500 2puffs
41	41	F	SevA	YES	45.8	NS	2.78	86	2.98	76	Salbutamol PRN die/UNIPHYL 600 mg, die/Fluticasone/Salmeterol Diskus 4puffs/day, 250 µg/puff/ Montelukast 10 mg, die
42	35	M	SevA	YES	32.4	NS	3.1	69	3.62	66	Salbutamol 100 µg die/ Fluticasone/Salmeterol Diskus 4 puffs/day, 250 µg/puff/ Montelukast 10 mg, die/ METHOTREXATE 5mg/week/ Tiotropium 18 µg, die
43	50	F	SevA	YES	40	NS	2.5	99	3.26	105	Salbutamol 100 µg, die/NASAL STEROIDS 200 µg hs/ ANTIHISTAMINE 20 ng, die/Fluticasone/Salmeterol Diskus 500 2puffs/day/ Montelukast 10 mg, die/ Diabeta/Glucophage/Micardis/Pantoloc/Calcium/Vitamin D
44	48	M	SevA	YES	33.3	NS	1.32	41	3.03	76	Salbutamol PRN die/QVAR 400 µg, die/ Fluticasone/Salmeterol 4puffs/day, 250 µg/puff/ Montelukast 10 mg,die/Lipitor/Hydrocholothiazide/Losartan
45	68	F	SevA	NO	31	EX S	1.66	73	2.28	78	Quetiapine 1 qhs /LIPITOR 20 mg qd/Salbutamol 100 µg, die/ Budesonide/Formoterol 8 puffs/day, 200 µg/puff/ NASAL STEROIDS 100 µg hs/Hydrocholothiazide/Losartan 100/25 mg qd/APO-OMEPRAZOLE qd
46	65	M	SevA	NO	27	NS	1.45	55	2.49	56	Salbutamol 100 µg, die/Fluticasone/Salmeterol 4 puffs/day, 250 µg/puff/ Tiotropium 18 mg, die
47	29	M	SevA	NO	27.1	NS	2.62	59	4.17	78	Salbutamol 100 µg, die/Fluticasone/Salmeterol Diskus 2 puffs/day
48	35	M	SevA	YES	N/A	NS	1.81	49	3.18	73	Terbutaline PRN die/ALVESCO 400 µg, die/Fluticasone/Salmeterol Diskus 2puffs/day, 500 µg/puff/ Tiotropium 18 µg,die
49	34	M	SevA	YES	30	NS	2.79	61	4.61	82	Alvesco 800 µg, die/Salbutamol 100 µg, die/Fluticasone/Salmeterol Diskus 2puffs/day, 500 µg/puff
50	35	M	SevA	YES	N/A	NS	1.45	40	2.72	63	Salbutamol/Alvesco 400 Mg, Die/Fluticasone/Salmeterol Diskus 2puffs/day, 500 mg/Puff

51	48	M	SevA	YES	N/A	NS	2.17	60	2.81	63	Salbutamol/Alvesco 400 Mg, Die/Fluticasone/Salmeterol Diskus 2puffs/day, 500 mg/Puff
52	73	M	COPD	NO	24.3	EX S	1.85	60	3.11	77	Salbutamol/Atrovent
53	59	M	COPD	NO	29.7	EX S	2.46	64	3.67	75	Salbutamol/Vioxx/Asa/Omeprazole/Atrovent
54	76	M	COPD	NO	31	EX S	1.46	50	2.69	69	Salbutamol/Amlopidine/Candesartan/Omeprazole
55	70	M	COPD	N/A	28.3	EX S	1.67	61	3.81	107	Salbutamol/Amlopidine/Novasen/Candesartan/Omeprazole
56	75	M	COPD	NO	27	EX S	1.7	62	3.08	86	Fluticasone Hfa/Salbutamol
57	55	F	COPD	N/A	25	S	1.85	73	2.85	95	Salbutamol
58	73	M	COPD	NO	21	S	1.05	35	2.69	68	Salbutamol/Combivent/Angiotensin Receptor Antagonist
59	71	M	COPD	N/A	28.3	EX S	0.67	37	2.49	108	Salbutamol/Tiotropium
60	58	M	COPD	NO	30.1	EX S	1.42	45	3.11	79	Salbutamol/Tiotropium/ Angiotensin Receptor Antagonist
61	61	M	COPD	YES	26.6	EX S	1	35	3.8	106	Salbutamol/Tiotropium

Table ST2. Differential cell count in induced sputum of severe asthmatic. The number of cells are expressed as percentage of total number of cells present in the sputum. The analysis refers to the induced sputum collected before (subjects 39,41-47) or earliest from the bronchoscopy (subjects 40, 48,49 and 51). Subject number 50 did not provide the sputum.

Subject	MACROPHAGES (%)	NEUTROPHILS (%)	LYMPHOCYTES (%)	EOSINOPHILS (%)	EPITHELIAL CELLS (%)
39	57.25	25.5	1.25	11.25	4.75
40	11	86.5	0	2.3	0.2
41	67.5	12.5	7.75	0.75	11.5
42	17	55	0	2	26
43	24.57	67.15	2	6.81	0.24
44	6	89.25	2	2.25	0,5
45	4.33	96	0	0	0
46	40.29	54.57	0.3	0.6	3.71
47	39.51	57.07	2	0	2
48	30.9	60.5	1.2	5.3	2
49	18.5	70	1.5	7	3
50	N/A				
51	12.2	51.5	1.2	24.2	10.7

Table ST3. Concentration of angiogenic factors (pg/ml) detected in the supernatant of HMVEC-L after 24 hours of incubation with IL-17A with respect to Vehicle treated cells. EGF, endoglin, BMP-9, leptin and Angiopoietin-2 were undetectable. Data are presented as means $\pm$ SEM (n=7 to 8), *p<0.05, **p<0.01, ***p<0.001

	Target	Vehicle	IL-17A 10ng/ml	IL-17A 100ng/ml	TNF- α 10ng/ml	PMA 40nM
HMVEC-L	CXCL8	160.1 $\pm$ 49.2	259.1 $\pm$ 82.2	332.0 $\pm$ 102.1 (**)	2361.9 $\pm$ 954.9 (**)	1841.8 $\pm$ 363.8 (*)
	G-CSF	106.3 $\pm$ 40.0	1001.3 $\pm$ 393.6	2584.4 $\pm$ 1035.6 (***)	4978.8 $\pm$ 3543.4 (*)	2300.1 $\pm$ 644.2 (*)
	Endothelin-1	429.0 $\pm$ 97.3	413.2 $\pm$ 88.7	440.8 $\pm$ 104.7	310.1 $\pm$ 38.0	24.8 $\pm$ 9.7 (*)
	FGF-1	16.5 $\pm$ 2.5	15.3 $\pm$ 2.8	16.1 $\pm$ 2.3	14.6 $\pm$ 2.7	22.1 $\pm$ 4.1
	Follistatin	1055.3 $\pm$ 540.0	1116.8 $\pm$ 503.2	1050.4 $\pm$ 454.5	396.1 $\pm$ 167.0	89.4 $\pm$ 18.2 (*)
	HGF	67.5 $\pm$ 16.6	63.7 $\pm$ 15.2	69.5 $\pm$ 15.8	121.1 $\pm$ 28.8 (*)	439.7 $\pm$ 74.7 (*)
	HB-EGF	114.7 $\pm$ 16.5	112.6 $\pm$ 15.6	118.0 $\pm$ 17.7	187.2 $\pm$ 26.6 (**)	579.7 $\pm$ 57.7 (*)
	PLGF	4044.8 $\pm$ 1715.0	4516.3 $\pm$ 2037.1	4236.4 $\pm$ 1742.7	2689.2 $\pm$ 1234.7 (*)	5270.9 $\pm$ 2801.2
	VEGF-A	324.9 $\pm$ 91.2	308.5 $\pm$ 90.5	317.3 $\pm$ 79.0	420.0 $\pm$ 124.3 (*)	181.2 $\pm$ 80.4
	VEGF-C	303.4 $\pm$ 121.1	287.8 $\pm$ 105.8	299.7 $\pm$ 116.0	527.8 $\pm$ 188.6 (*)	350.6 $\pm$ 137.3
	VEGF-D	12.8 $\pm$ 2.3	12.1 $\pm$ 2.5	12.7 $\pm$ 2.6	15.1 $\pm$ 1.7	15.5 $\pm$ 2.2 (*)
	FGF-2	1500 $\pm$ 247.4	1514 $\pm$ 248.4	1550.1 $\pm$ 239.4	1798.3 $\pm$ 313.3 (*)	1801.4 $\pm$ 171.3 (*)

Supporting informations

Interleukin-17A and vascular remodeling in severe asthma; lack of evidence for a direct role.

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Methods:

Bronchial epithelial, fibroblast and airway smooth muscle cell culture

Normal human bronchial epithelial (NHBE) cells were also obtained from lungs of donors. After digestion in Pronase (1.5mg/ml) (Sigma Aldrich) for 16 hours, micro-dissected tissue was dissolved in DMEM containing Anti-Anti. The following day, tissues were rinsed with Ham's F12 medium (Thermo Fisher) supplemented with 20% FBS and Anti-Anti. After 3 washes, undigested tissue was discarded. Cell suspensions were pooled, and centrifuged. Resulting cells were cultured in BEGMTM Medium (human basal epithelial medium (BEBM) supplemented with GF (Bovine Pituitary Extract, hydrocortisone, human epidermal growth factor, epinephrine, transferrin, insulin, retinoic acid, triiodothyronine, and gentamicin/amphotericin-B; Lonza).

Primary human airway smooth muscle cells (ASMCs) were cultured from transplant grade lungs procured by the International Institute for the Advancement of Medicine. Protocols were approved by an Institutional Review Board. After digestion in collagenase (0.4mg/ml) for 16 hours, the tissue was dissolved in Dulbecco's Modified Eagle's Medium (DMEM) containing streptomycin, penicillin and amphotericin B (Anti-Anti) (Thermo Fisher Scientific). Cells were maintained in DMEM supplemented with Anti-Anti and 10% fetal bovine serum (FBS) (Thermo Fisher Scientific). Medium was replaced every second day. For long term storage, cells were cryo-stored in 90% FBS:10% dimethyl sulfoxide (DMSO; Sigma Aldrich) in liquid nitrogen.

NHBE were isolated as previously described [1] cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin.

Cell viability assay

HMVEC-L were seeded in 96-well plate at a density of 5×10^3 cells/well, serum starved in EBM-2 + GF + 0.5% FBS for 16 hours and then exposed to IL-17A (10-100 ng/ml) (R&D) or vehicle (4 mM HCl in PBS). Twenty four hours before the end of the treatment, 10 μ L of MTT (Sigma Aldrich) solution (5 mg/mL in PBS) was added to each well and then incubated for 4 hours in a humidified atmosphere of 95% air -5% CO₂ at 37°C. Next, MTT solution was removed and 100 μ L

dimethylsulfoxide (Sigma Aldrich) was added to each well to solubilize formazan crystals. The absorbance was read at 570 nm on a microplate reader (Infinite® M1000 PRO; TECAN).

Analysis of the production of angiogenic factors

Sputum and cell supernatant were processed for the analysis of the expression of angiogenic factors by a bead-based suspension array using the Luminex xMAP technology (Millipore) following manufacturer's instructions. The factors detectable by the array and relative range of detection are the following:

- 13.7 - 10,000 pg/mL: Angiopoietin-2, Fibroblast growth factor (FGF)-1 and -2 (or bFGF), granulocyte colony stimulating factor (G-CSF), vascular endothelial growth factor (VEGF)-A
- 2.7 - 2,000 pg/mL: Bone morphogenetic protein (BMP)-9, epithelial growth factor (EGF), Endothelin-1
- 27.4 - 20,000 pg/mL: Endoglin, Follistatin, hepatocyte growth factor (HGF)
- 1.4 - 1,000 pg/mL: Heparin-binding EGF-like growth factor (HB-EGF), Placental growth factor (PLGF)
- 137.2 - 100,000 pg/mL: Leptin
- 6.9 - 5,000 pg/mL: VEGF-C, VEGF-D

Analysis of mRNA expression

Sequences of primer pairs were as follows: CXCL8 (Forward: 5'-GTGCAGTTTTGCCAAGGAGT-3', Reverse: 5'-CTCTGCACCCAGTTTTCCTT-3'); G-CSF (Forward: 5'-CACTCTGGACAGTGCAGGAA-3', Reverse: 5'-TGTAAGGTGGCACACAGCTTC-3'); VEGF-A (Forward: 5'-CCTTGCTGCTCTACCTCCAC, Reverse: 5'-TGGTGATGTTGGACTCCTCA-3') and GAPDH (Forward: 5'-AGCAATGCCTCCTGCACCACC-3', Reverse: 5'-CCGGAGGGGCCATCCACAGTC-3'). Reactions containing water or cDNA synthesized without reverse transcriptase, as template, were used as negative controls, which resulted in no PCR products. Reactions were performed in duplicate and analyzed via relative quantitation with data presented as fold change compared to vehicle-treated cells after normalization to GAPDH.

miRNA expression profiling: Total RNA was extracted by HMVEC-L treated for 12 h with IL-17A 100 ng/ml or vehicle alone with miRCURY™ RNA Isolation Kits - Cell & Plant (EXIQON). After RNA quality control by Agilent2100 bioanalyzer, the samples were processed for miRCURY LNA microRNA Hi-Power labeling and analyzed by miRCURY LNA microRNA array 7th Generation (Exiqon).

References

1. Baglole CJ, Reddy SY, Pollock SJ, Feldon SE, Sime PJ, Smith TJ, Phipps RP. Isolation and phenotypic characterization of lung fibroblasts. *Methods Mol Med* 2005; 117: 115-127.