

ORIGINAL ARTICLE Basic Mechanisms in Allergic Disease

Interleukin-25 promotes basic fibroblast growth factor expression by human endothelial cells through interaction with IL-17RB, but not IL-17RA

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Summary

Background Unlike other IL-17 family members, the Th2-derived cytokine IL-25 (IL-17E) induces (promotes) Th2 responses. One or both of the two receptors for IL-25 (IL-17RA, IL-17RB) is expressed on inflammatory cells and tissue structural cells, suggesting that in addition to promoting Th2-type inflammation IL-25 may also act on structural cells at sites of Th2-type inflammation such as in the asthmatic bronchial mucosa to promote remodelling changes.

Objective Our previous studies showed elevated expression of IL-25 and IL-17RB immunoreactivity in asthmatic airways with co-localization of the latter to endothelial cells. We therefore hypothesized that IL-25 acts on endothelial cells through this receptor to induce production of the key angiogenic and remodelling cytokine basic fibroblast growth factor (bFGF).

Methods Polymerase chain reaction (PCR) immunocytochemistry/immunohistochemistry and ELISA were employed to detect expression of IL-17RB, IL-17RA and bFGF by human vascular endothelial cells (HUVEC) and immunoreactivity for IL-25 and bFGF in asthmatic bronchial biopsies. Receptor-blocking antibodies, PCR and an *in vitro* angiogenesis assay were used to investigate whether IL-25 acts on IL-17RB or IL-17RA to induce bFGF expression and angiogenesis. PCR was also employed to investigate the signalling pathways involved in IL-25-mediated bFGF expression.

Results HUVEC constitutively expressed IL-17RB, IL-17RA and bFGF. Production of the latter was further increased by IL-25, but attenuated after blockade of the IL-17RB, but not the IL-17RA receptor. Neutralization of endogenous VEGF and bFGF completely abrogated IL-25-induced angiogenesis which was also inhibited by blocking IL-17RB, but not IL-17RA. The PI3K-specific inhibitor LY294002 also completely attenuated IL-25-induced bFGF expression. Immunoreactivity for IL-25 and bFGF was elevated in the asthmatic bronchial mucosa and the expression of each correlated with the other.

Conclusions and Clinical Relevance Our data support the hypothesis that IL-25 contributes to elevated bFGF in asthmatic airways by acting on the endothelial cell IL-17RB receptor through PI3K-signalling pathways. Targeting the pathways might benefit therapy of airways remodelling.

Keywords basic fibroblast growth factor, endothelial cells, IL-25, IL-25 receptor, remodelling

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Introduction

Interleukin 25 (IL-25), or IL-17E, is a 'distant' member of the IL-17 cytokine family (IL-17A-F), with less homology and distinct biological functions. In particular, unlike other family members, IL-25 augments Th2-type T cell immune responses [1, 2]. In mice, exogenous administration of IL-25 [1, 3] or transgenic expression [4, 5] induces Th2-type asthma-like inflammation in the airways. Conversely, anti-IL-25 antibody reduces airways inflammation in animal 'models' of asthma [6, 7]. Aside from T cells, epithelial cells, mast cells, basophils and eosinophils may potentially produce IL-25 [8, 9].

The principal recognized IL-25 receptor (IL-25R, IL-17RB) is a 56-kDa single transmembrane protein. Binding of IL-25 to this receptor triggers a range of effects on target cells which include eosinophils, mast cells and monocytes, as well as T cells [8, 10–12]. Although IL-25 exerts a majority of its cellular effects by acting on IL-17RB, Rickel and colleagues recently produced data from animals and human peripheral blood mononuclear cells suggesting that the IL-25 receptor may act as a functional complex of IL-17RA and IL-17RB [13]. Similarly, it has also been suggested that IL-17RA is a functional component of the IL-17A and IL-17F receptors [14].

Tissue remodelling (healing, lay down of fibrous/scar tissue, angiogenesis and mucous and smooth muscle hyperplasia) is a feature of many chronic inflammatory responses to tissue injury, whether they are injury/repair responses, infective or inflammatory insults or neoplastic processes. IL-25R (IL-17RB) is expressed on many structural cells involved in these processes such as smooth muscle and fibroblasts [15, 16]. We recently observed expression of IL-25R on blood vessel endothelial cells in the bronchial mucosa and the skin [17, 18], allowing the hypothesis that IL-25 exerts a wider range of effects on tissue remodelling than hitherto recognized. We are exploring the possibility that IL-25 acts on a range of structural cells to increase their production of remodelling cytokines and growth factors. In this particular study, we focus on basic fibroblast growth factor (bFGF), a key remodelling mediator which exhibits a wide array of effector activities including cellular proliferation, differentiation, survival and motility, neurogenesis, angiogenesis and smooth muscle hypertrophy [19]. Known sources include epithelial cells, endothelial cells, neurons and inflammatory cells such as mast cells, eosinophils, T cells and macrophages [19]. As bFGF is known to be over expressed in the asthmatic bronchial mucosa [20] and has been implicated in regulating angiogenesis at this site [21], and bearing in mind our recent demonstration that endothelial cells express IL-25 receptors [17], we hypothesized

that IL-25 induces bFGF production by endothelial cell targets by acting on the 'classical' IL-17RB, but not the IL-17RA receptor.

Methods

Bronchial biopsies

This study was approved by one of the Ethics Committees of King's College London School of Medicine (that of Guy's Hospital). Each subject provided written, informed consent. The patients were recruited in the Department of Asthma, Allergy and Respiratory Science, King's College London School of Medicine. Endobronchial biopsies were obtained from 20 asthmatics and 20 normal controls as previously described [17, 21].

Medium and reagents

All reagents and human vascular endothelial cells (HUVEC; pooled from three different donors) were purchased from PromoCell GmbH (Heidelberg, Germany) unless otherwise specified.

Culture of human vascular endothelial cells

Cells were used for experiments prior to their forth passage. Briefly, cells (0.5×10^6 per well) were seeded in six-well plates (Nunc A/S, Roskilde, Denmark) pre-coated with gelatin (0.5%; Sigma, Poole, UK) in complete medium (endothelial cell growth medium kit; PromoCell GmbH) and grown to approximately 80% confluence in a 5% CO₂ incubator at 37°C. The cells were then exposed to various stimuli including recombinant human (tumour necrosis factor- α , TNF- α ; R&D Systems), IL-17 Abingdon, UK and IL-25 (R&D Systems) and others (see below). To identify IL-25R immunoreactivity on HUVEC, some were seeded and cultured to approximately 80% confluence in chamber slides (Nalge Nunc International, Naperville, IL, USA) for 48 h. At different time points as shown in the Results section, cells were harvested for further investigation.

Immunostaining of IL-25R (17RB) and IL-17RA

Immunostaining for IL-25R (IL-17RB) and IL-17RA on HUVEC was performed according to standard methodology as previously described [17]. Briefly, HUVEC were cultured on chamber slides for 24 h as above then fixed in 4% paraformaldehyde/PBS for 10 min. After rinsing in 3% H₂O₂ for 15 min and in PBS for 10 min, the chamber slides were incubated with murine monoclonal antibodies against IL-17RA (MAB177, 1/50; R&D

Systems) and IL-17RB (LB-2044, 1/50; Autogene Bioscience, Wiltshire, UK) overnight, respectively. After washing with PBS, the slides were incubated with rabbit anti-mouse IgG (1/100; Dako, Cambridgeshire, UK) for 30 min, washed in PBS and then incubated with mouse peroxidase-anti-peroxidase complex (1/50; Jackson ImmunoResearch Lab, Inc., PA, USA). Positive signals were visualized (brown colour) with 3, 3'-Diaminobenzidine (DAB; Sigma). Substitution of the primary antibody with an irrelevant isotype-matched immunoglobulin (mouse IgG1; Sigma) was used as a negative control.

RNA extraction, semi-quantitative polymerase chain reaction (PCR) and real-time PCR

Total cellular RNA was extracted from HUVEC using the RNeasy Mini kit (QIAGEN, West Sussex, UK), and reverse transcribed using GoTaq Hot Start Polymerase (Promega Ltd, Southampton, UK). PCR amplification was performed with 35 cycles of 3 min at 95°C, 60 s at 50°C and 50 s at 72°C. Oligonucleotide primers were 5'-GGA GGA GCA GCG GCA GTC A-3' (forward sequence) and 5'-TGT CCC AGC CCG AGT TCT TC-3' (reverse sequence) for human IL-17RA, 5'-CCT TCC CGG ACT GGT TCA TTG-3' (forward sequence) and 5'-AAG CCC CAG TCC CAG GTT TAC-3' (reverse sequence) for human IL-25R (IL-17RB), 5'-GCC TTC CCG CCC GGC CAC TTC AAG G-3' (forward sequence) and 5'-GCA CAC ACT CCT TTG ATA GAC ACA A-3' (reverse sequence) for human bFGF and 5'-TCT CAG CCC TCT TCA AAA ACT TCT C-3' (forward sequence) and 5'-ATG ACT TCC AAG CTG GCC GTG GCT-3' (reverse sequence) for human IL-8. PCR products were resolved on 2% agarose gels, stained with ethidium bromide and photographed. To assess the expression of IL-25R, IL-17RA and bFGF mRNA in HUVEC, gels were scanned using a Fluor-S Max Multi-Imager and analysed by Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA). Human GAPDH from the same samples (5'-CCA CCC ATG GCA AAT TCC ATG GCA-3' and 5'-AGA CCA CCT GGT GCT CAG TGT AGC-3') was amplified as an internal control to correct for total loading.

Real-time PCR was further used to quantify IL-25-induced bFGF mRNA expression in HUVEC stimulated with different concentrations of IL-25 using iQTM SYBR Green Supermix (Bio-Rad Co. Ltd, CA, USA). Specific bFGF primers were the same as for RT-PCR as above. Human GAPDH mRNA was used as the endogenous control gene. All measurements were performed in triplicate. After denaturation at 94°C for 3 min, amplification conditions were 45 cycles of denaturation at 94°C for 40 s, annealing at 55°C for 40 s and synthesis at 72°C for 50 s and an extension for 5 min at 72°C. Dynamic changes in bFGF transcription were analysed using Opticon Monitor Version 3.1 software (Bio-Rad Co. Ltd).

bFGF ELISA

Supernatants were collected from HUVEC cultured with or without IL-25 (1 ng/mL) for 24, 48 and 72 hs. ELISA kits were employed to measure human bFGF according to the manufacturer's instructions (RayBiotech, Inc., Norcross, GA, USA). Limit of detection was 50 pg/mL.

Blockade of IL-25 receptors

Human vascular endothelial cells were pre-incubated with monoclonal (MAB1771, 10 µg/mL; R&D Systems) and polyclonal (AF177, 10 µg/mL; R&D Systems) neutralizing antibodies against IL-17RA according to the manufacturer's instructions and monoclonal (XX19R, 10 µg/mL; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and polyclonal (AP17497PU-N, 10 µg/mL; Acris Antibodies GmbH, Herford, Germany) antibodies against IL-17RB for 30 min prior adding recombinant human IL-25 (1 ng/mL; R&D Systems) for a further 24 h of incubation. Cells were then harvested and total RNA extracted to detect bFGF mRNA expression.

To further evaluate whether these antibodies could block IL-17 or IL-25-induced activities on endothelial cells, we further tested their abilities to block IL-17 and IL-25-induced IL-8 mRNA expression (see above).

In vitro angiogenesis assay

The effects of IL-25 on angiogenesis were investigated using an *in vitro* angiogenesis assay (AngioKit; TCS CellWorks, Buckingham, UK) as previously described [17]. To evaluate whether VEGF and bFGF are involved in IL-25-induced angiogenesis, recombinant human VEGF (R&D Systems, 10 ng/mL), human bFGF (10 ng/mL; Millipore Ltd, Watford, UK), human IL-25 (10 ng/mL; R&D Systems), and monoclonal neutralizing antibodies against human VEGF (5 µg/mL; R&D Systems) and human bFGF (bFM-1, 5 µg/mL; Millipore, Ltd) were added to assay culture wells, either alone or combination, on day 0 and replenished on days 4, 7 and 9. In addition, antibodies against IL-17RA and IL-17RB (as above) were added to further wells to confirm which receptor plays a role in IL-25-induced angiogenesis. On day 11, cultured cells were fixed and vascular structures visualized by labelling with mouse anti-CD31 as previously described [17].

IL-25 signalling leading to bFGF expression by HUVEC

A number of signalling inhibitors were examined for their effects on IL-25-induced bFGF mRNA expression by HUVEC including SB203580 [p38 mitogen-activated protein kinase (p38 MAPK) inhibitor, 10.0 µM, Promega Ltd], U0126 [mitogen-activated protein/extracellular

signal-regulated kinase 1 and 2 (MEK1 and MEK2) inhibitor, 10.0 μM , Calbiochem Corp., La Jolla, CA, USA], LY294002 [phosphatidylinositol 3-kinase (PI3K) inhibitor, 10.0 μM ; Merck Chemicals Ltd, Nottingham, UK], PD98059 MEK1 inhibitor, 5.0 μM , Cayman Chemical Company, USA, and Bay11-7082 [nuclear transcription factor kappa B (NF- κB) inhibitor; 2.5 μM , Cayman Chemical Company]. The optimal concentrations of these inhibitors were established previously [17]. HUVEC at passage 3 were incubated with IL-25 and the inhibitors for 24 h. The cells were then harvested, cellular viability assessed by Trypan blue exclusion and cellular total mRNA isolated as above. The effects of the inhibitors on IL-25-induced bFGF expression were analysed by PCR.

To further investigate whether IL-25 signalling in HUVEC involves the PI3K pathway, phosphorylation of PI3K was detected by Western blotting. Briefly, HUVEC cultured with or without IL-25 (1 ng/mL) were harvested and washed with ice-cold PBS and then lysed in 50 μL lysis buffer (25 mM HEPES, 1.5% Triton X-100, 0.1% SDS, 0.5 M NaCl, 5 mM EDTA, 0.1 mM sodium deoxycholate; Sigma-Aldrich Company Ltd, Poole, UK) containing a protease inhibitor cocktail (Roche, Welwyn, UK) and sodium orthovanadate (10 mM, Sigma-Aldrich). Cell debris was removed by centrifugation at 15,000 g for 20 min at 4°C. The supernatants were

boiled in 6 $\times$ Laemmli sample buffer (Bio-Rad Laboratories Ltd, Hertfordshire Company Ltd, UK) for 6 min. The protein concentrations in the supernatants were determined using the BCA (bicinchoninic acid) protein assay kit (Thermo Fisher Scientific, Northumberland, UK). Equal amounts of protein (20 μg protein per lane) were subjected to electrophoresis and then transferred to nitrocellulose membranes and incubated in blocking buffer (0.05% Tween-20, pH 7.4, 5% fat-free milk) for 1 h at room temperature. Antibodies against total PI3K p85 (19H8) and p-PI3K p85/p55 (Tyr458/Tyr199) (1/1000) were purchased from Cell Signalling Technology Inc. (Danvers, MA, USA). Total GAPDH was detected with a polyclonal antibody (WH0002597M1, 1/5000; Sigma) as the loading control. In addition, phosphorylation of ERK, p38MAPK and NF- κB was also detected as previously described [17]. The positive signals were subsequently developed with Western blotting detection reagents (Amersham, Little Chalfont, UK) according to the manufacturer's instructions.

Immunohistochemistry for IL-25 and bFGF in the bronchial mucosa

A mouse anti-human IL-25 (MAB 1258, 1/20; R&D Systems) and a rabbit anti-human bFGF (ab106245,

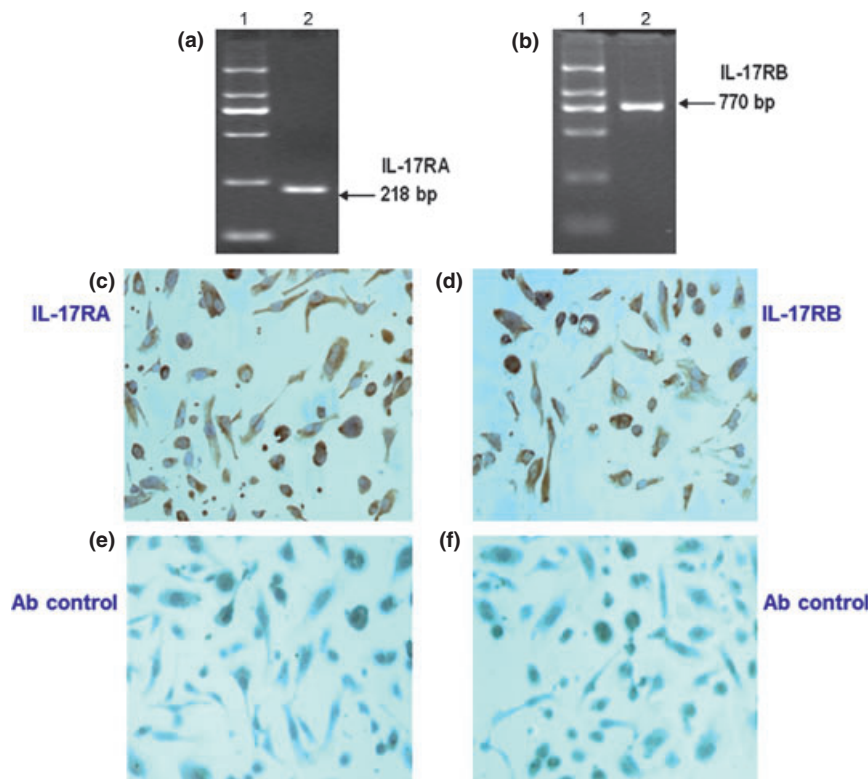


Fig. 1. Expression of IL-17RA (a) and IL-17RB (b) mRNA by unstimulated human vascular endothelial cells (HUVEC) cultured for 24 h in 6-well plates. Immunostaining for IL-17RA (c) and IL-17RB (d) in HUVEC cultured in chamber slides with specific antibodies or isotype-matched control antibody (e, f). Original magnification $\times 10$.

1/100; Abcam, Cambridge, UK) were employed to detect immunoreactivity for IL-25 and bFGF in bronchial biopsies as previously described [17, 21]. Positively staining cells were detected with Fast Red (Sigma).

Statistical analysis

Data were analysed with the statistical package GraphPad Prism 4 (GraphPad Software, La Jolla, CA, USA). The *in vitro* data were analysed by ANOVA followed by Student's *t*-test. The Mann–Whitney *U*-test was used to compare IL-25 and bFGF immunoreactivity in bronchial biopsies. For all tests, $P < 0.05$ was considered significant.

Results

HUVEC expressed IL-17RA and IL-17RB

RT-PCR and immunocytochemistry showed that HUVEC constitutively expressed both IL-17RA (Fig. 1a and c) and IL-25R (IL-17RB) (Fig. 1b and d) mRNA and protein.

IL-25 increased expression of bFGF mRNA and protein in HUVEC

Exposure of HUVEC to IL-25 significantly induced expression of mRNA encoding bFGF in a concentration-dependent fashion ($P < 0.05$) (Fig. 2a). This effect was comparable with that of TNF- α employed as a positive control. ELISA showed that culture of HUVEC with IL-25 significantly increased supernatant concentrations of bFGF compared with culture medium control at each time-point (Fig. 2b). Expression continued to increase up to 72 h ($P < 0.01$). The effects of TNF- α used as positive control were similar. At these concentrations, the combination of IL-25 and TNF- α showed no additive or synergistic effect on bFGF expression by endothelial cells (Figs. 2c and d). IL-13 alone did not enhance bFGF production and did not alter the effect of IL-25 in this regard (Figs. 2c and d).

IL-25-induced angiogenesis in vitro

In an *in vitro* angiogenesis assay, IL-25 enhanced spontaneous lengthening and branching of microvascular tubules by HUVEC in a manner comparable to the

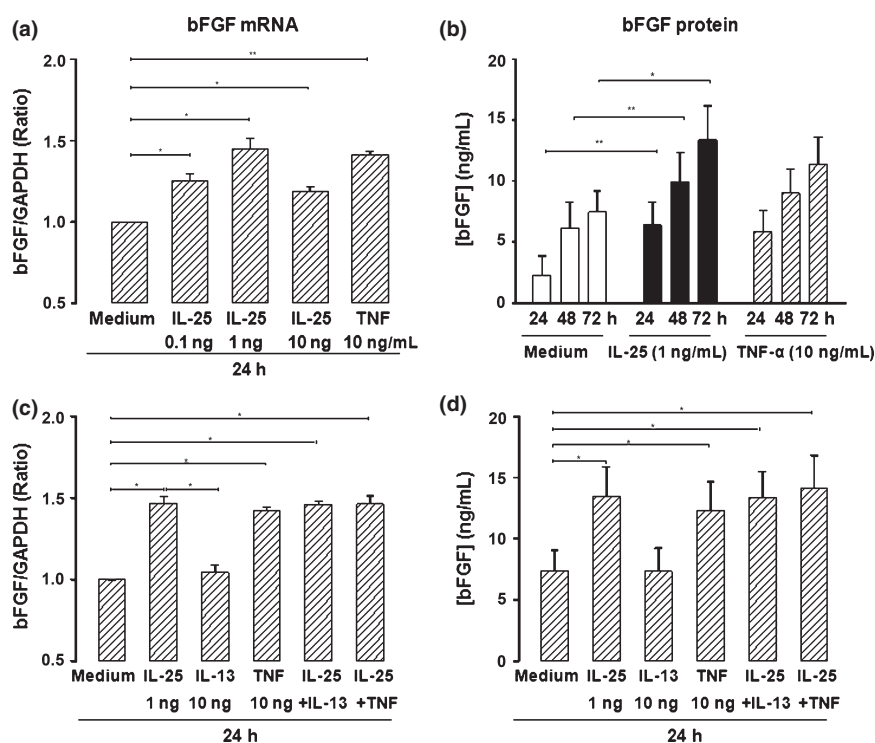


Fig. 2. (a) Effects of IL-25 (0.1, 1, 10 ng/mL), tumour necrosis factor- α (TNF- α ; 10 ng/mL) and medium alone on basic fibroblast growth factor (bFGF) mRNA expression by human vascular endothelial cells (HUVEC) (24 h stimulation) as measured by real-time PCR. Data are expressed as the bFGF/GAPDH ratio ($n = 4$). (b) bFGF concentrations in the supernatants of HUVEC cultured for varying periods (24 h, 48 and 72 h) with medium control, IL-25 1 ng/mL and TNF- α 10 ng/mL ($n = 5$ separate experiments performed in triplicate). Effects of IL-25, IL-13 and TNF- α alone or in combination on expression of bFGF mRNA (c) (24 h, $n = 4$) and protein (d) ($n = 5$). Bars show the mean $\pm$ SEM. Student's paired *t*-test, * $P < 0.05$, ** $P < 0.01$.

patterns induced by exogenous recombinant VEGF and bFGF (Figs. 3a and b). Blockade of both VEGF and bFGF with neutralizing antibodies partially and additively inhibited spontaneous microtubule formation and completely attenuated the enhancement provided by IL-25 (Fig. 3a and b, Table 1).

Blockade of IL-17RB, but not IL-17RA abrogated IL-25-induced bFGF expression in HUVEC

Prior blockade of IL-17RA with adequate concentrations of two commercially available monoclonal and polyclonal anti-IL-17RA-blocking antibodies [13] did not attenuate IL-25-induced bFGF mRNA expression (Fig. 4a). On the other hand, commercially available monoclonal and polyclonal anti-IL-17RB antibodies, although not previously described to block the receptor, significantly attenuated IL-25-induced bFGF mRNA expression by HUVEC (Fig. 4b). To further confirm the activities of these antibodies, we tested their effects on the known ability of IL-17 to induce IL-8 expression in HUVEC [22]. Q-PCR analysis showed that both anti-IL-17RA antibodies used in this study completely abrogated IL-17-induced IL-8 mRNA expression (Fig. 4c). We also documented using Western blotting, inhibition of IL-25-induced TCt-1 activation, recruited to the SEFIR domain of IL-17RA, by these antibodies at the same concentration (data not shown). In contrast to

IL-17, IL-25 inhibited IL-8 mRNA expression in endothelial cells, an effect which was reversed by blocking IL-17RB (Fig 4d). These observations suggest that all of the antibodies against IL-17RA and IL-17RB used in this study produce functional blockade of their respective receptors and that functional blockade of IL-17RA did not abrogate IL-25-induced bFGF expression.

Blockade of IL-17RB, but not IL-17RA attenuated IL-25-induced angiogenesis in vitro

In the *in vitro* angiogenesis assay, blockade of IL-17RB, but not IL-17RA reduced IL-25-induced vascular junctions, tubules and tubule elongation by more than 80% (Figs. 5a and b).

Effects of signalling inhibitors on IL-25-induced bFGF mRNA expression by HUVEC

Human vascular endothelial cells were co-incubated with IL-25 and a range of signalling inhibitors at pre-determined concentrations for 24 h. Real-time PCR analysis (Fig. 6a) showed that the PI3K inhibitor LY294002 not only completely abrogated IL-25-induced bFGF mRNA expression but also reduced it below 'baseline' production observed in the presence of medium control. On the other hand, the p38 MAPK inhibitor SB203580, the MEK1 inhibitor PD98059, the MEK 1/2

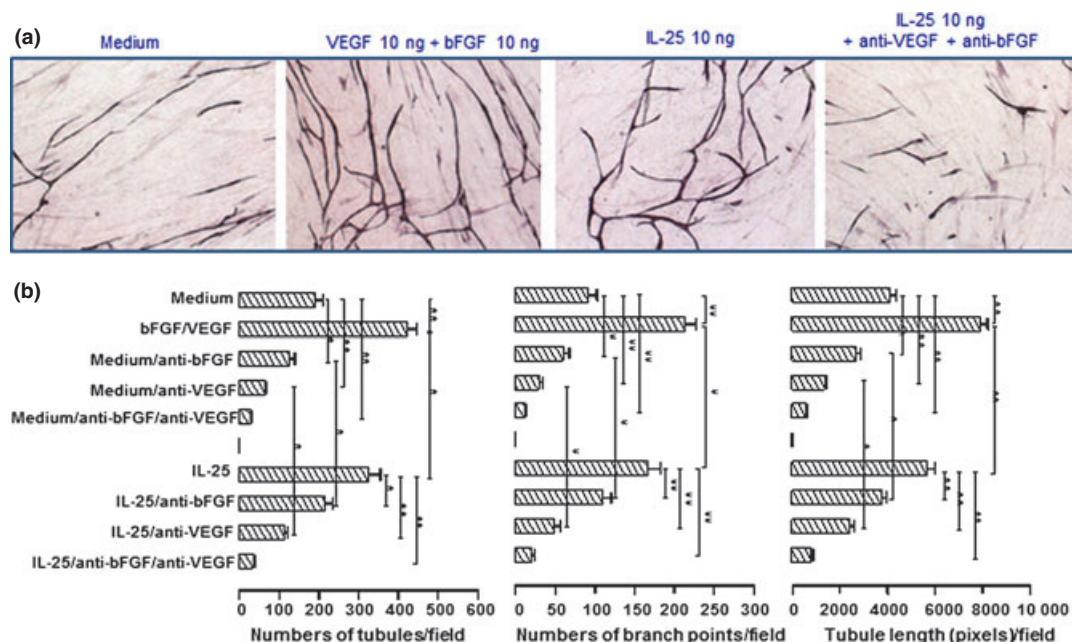


Fig. 3. (a) Top panel: representative light photomicrographs ($\times 4$ original magnification) (from left to right) show formation of primitive vascular tubule structures by human vascular endothelial cells after 11 days in culture with medium, vascular endothelial growth factor (VEGF) basic fibroblast growth factor (bFGF) (each 10 ng/mL), IL-25 (10 ng/mL) and IL-25 (10 ng/mL) + monoclonal anti-VEGF and anti-bFGF (5 μ g/mL). (b) Bottom panel (from left to right): computer-assisted quantification of numbers, branch points and total tubule lengths. Bars show the mean $\pm$ SEM of five separate experiments performed in duplicate. Student's paired *t*-test, **P* < 0.05, ***P* < 0.01.

Table 1. Effects of bFGF and VEGF on IL-25-mediated angiogenesis*

	Tubules	Branch points	Tubule length
% Enhancement			
bFGF + VEGF	120.8%	135.6%	93.8%
% Inhibition			
Medium + anti-bFGF	34.0%	33.2	33.8%
Medium + anti-VEGF	67.0%	67.3%	66.7%
Medium + anti-bFGF/VEGF	86.0%	85.3%	86.5%
% Enhancement			
IL-25	70.2%	83.0%	38.9%
% Inhibition			
IL-25/anti-bFGF	34.1%	33.9%	34.3%
IL-25/anti-VEGF	64.6%	70.5%	57.2%
IL-25/anti-bFGF/VEGF	89.5%	87.9%	86.0%

*Results are expressed as the mean percentages of enhancement by cytokines or inhibition by the antibodies used compared with medium alone or IL-25.

inhibitor U0126 and the NF- κ B inhibitor Bay 11-7082 showed no significant inhibitory effects under the conditions employed. Cellular viability remained > 99% throughout all experiments. Western blotting (Fig. 5b) showed that IL-25-induced activation of a number of signalling factors, including ERK, p38MAPK and NF- κ B as previously reported [17]. We also confirmed that exposure of HUVEC to IL-25 was accompanied by rapid

phosphorylation of PI3K, maximal within approximately 30 min of exposure and then slightly declining by 60 min (Figs. 6c and d). Anti-IL17RB, but not anti-IL-17RA, blocked IL-25-induced PI3K activation (Fig. 6c).

Immunoreactivity for IL-25 and bFGF in bronchial biopsies

The median numbers of cells showing immunoreactivity for IL-25 and bFGF were significantly elevated in the bronchial mucosa of the asthmatics compared with the controls (Fig. 7). In the asthmatics, the numbers of IL-25 immunoreactive cells correlated positively with the numbers of bFGF immunoreactive cells ($r = 0.538$, $P = 0.014$).

Discussion

Our data clearly suggest that in addition to its well-described effects in propagating and perpetuating Th2 T cell-driven inflammatory responses, IL-25 may also play a role in tissue remodelling at sites of inflammation. In a previous study, we showed that IL-25 induces expression of VEGF and VEGF receptors by human endothelial cells, suggesting a possible mechanism for IL-25-induced angiogenesis [17]. In that study,

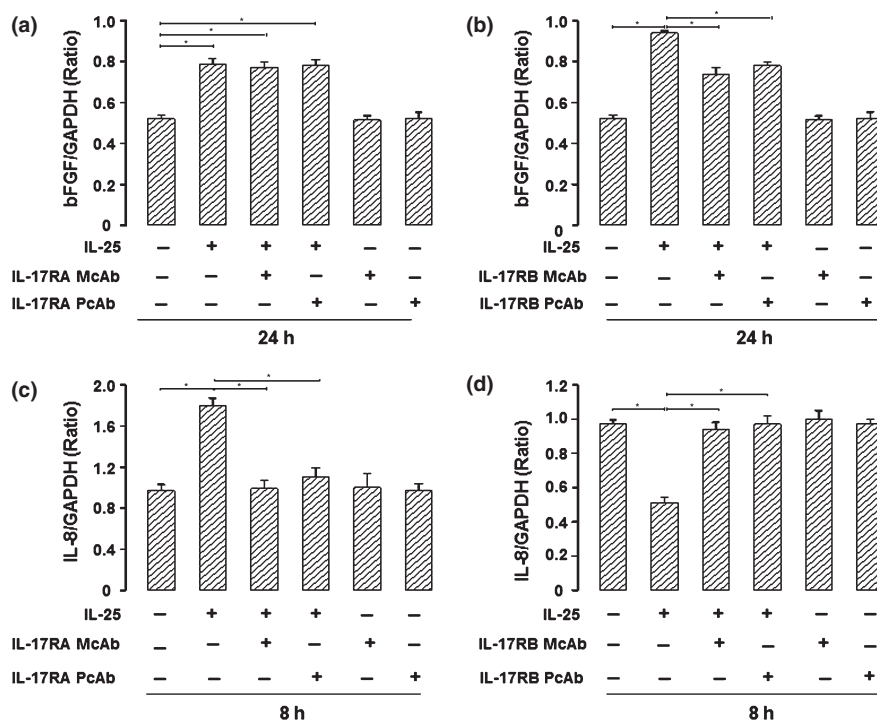


Fig. 4. (a) Top panel: Effects of blockade of IL-17RA (left) and IL-17RB (right) on IL-25 (1 ng/mL, 24 h) induced bFGF mRNA expression by human vascular endothelial cells (HUVEC). HUVEC were pre-incubated for 30 min with anti-IL-17RA or IL-17RB monoclonal (Mc-Ab) or polyclonal (Pc-Ab) antibodies (10 μ g/mL in each case) prior to adding IL-25 as described in the Methods. (b) Bottom panel: Effects of blockade of IL-17RA (left) and IL-17RB (right) on IL-17 (10 ng/mL, 8 h) (left) and IL-25 (1 ng/mL, 8 h) (right) induced IL-8 mRNA expression. Bars show the mean $\pm$ SEM ($n = 5$). Student's paired t -test, * $P < 0.05$.

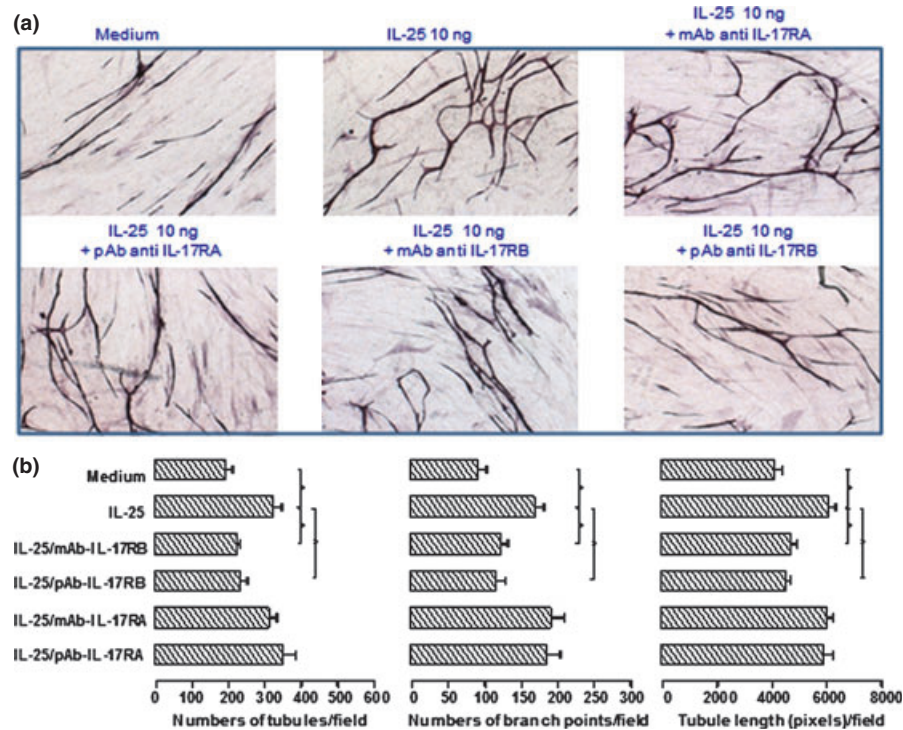


Fig. 5. (a) Top panel: representative light photomicrographs ($\times 4$ original magnification) showing formation of primitive vascular tubule structures by human vascular endothelial cells after 11 days in culture with medium, IL-25 (10 ng/mL) and IL-25 (10 ng/mL) with monoclonal or polyclonal antibodies (10 μ g/mL) against IL-17RA or IL-17RB. (b) Bottom panel: computer-assisted quantification of numbers, branch points and total tubule lengths. Bars show the mean $\pm$ SEM of five separate experiments performed in duplicate. Student's paired *t*-test, **P* < 0.05.

however, neutralization of VEGF attenuated only about 70% of IL-25-induced angiogenesis, suggesting that IL-25 might also induce production of other vascular growth factors. Following on from this, we here show that IL-25, acting through IL-17RB, also induces endothelial cells to produce bFGF, a remodelling cytokine with the potential not only to induce angiogenesis but also to cause fibrosis and smooth muscle hyperplasia at sites of chronic inflammation [19]. In our *in vitro* model, neutralization of both VEGF and bFGF attenuated spontaneous angiogenesis and completely abrogated IL-25-enhanced angiogenesis, suggesting that both factors together mediate this effect of IL-25 (although the possibility of very small effects by other mediators cannot be excluded). This is further supported by our observed positive correlation between IL-25 and bFGF immunoreactivity in the asthmatic bronchial mucosa. The bell-shaped concentration response curve for IL-25 in inducing expression of bFGF is compatible with the hypothesis that higher concentrations of IL-25 induce IL-25R internalization, a phenomenon we observed in our previous report on IL-25-induced VEGF expression [17] and which has been described for other cytokines and chemokines and their receptors, such as IL-5 and eotaxin [22, 23]. Other studies [15, 16] suggest that other structural cells such as smooth muscle cells

and fibroblasts also express IL-25 receptor, raising the possibility that IL-25 exerts even wider ranging remodelling effects. It is interesting that other IL-17 family members (such as IL-17A) have been reported to exert similar effects on endothelial cells [24–26] although through different receptors (IL-17RA and IL-17RC) and different signalling pathways (MAPK).

Our data also confirm that HUVEC constitutively express receptors for both IL-25 (IL-17RB) and IL-17 (IL-17RA). Although it has been reported that the biological effects of IL-25 are mediated through its specific high-affinity receptor IL-17RB [27, 28], Rickel et al. [13] recently showed that unlike those from wild-type mice, cultured splenocytes from both IL-17RB and IL-17RA gene deleted mice did not produce IL-5 or IL-13 in response to IL-25 stimulation or develop airways inflammation following intranasal administration of IL-25. Furthermore, treatment with blocking monoclonal antibodies against either IL-17RB or IL-17RA completely abrogated IL-25-induced airways inflammation in naïve BALB/c mice, similarly to the effects of an IL-25-blocking antibody, suggesting that IL-25-mediated activities might require interaction with both IL-17RA and IL-17RB. In contrast, we found that blockade of IL-17RB, but not IL-17RA, significantly attenuated IL-25-enhanced bFGF mRNA expression by

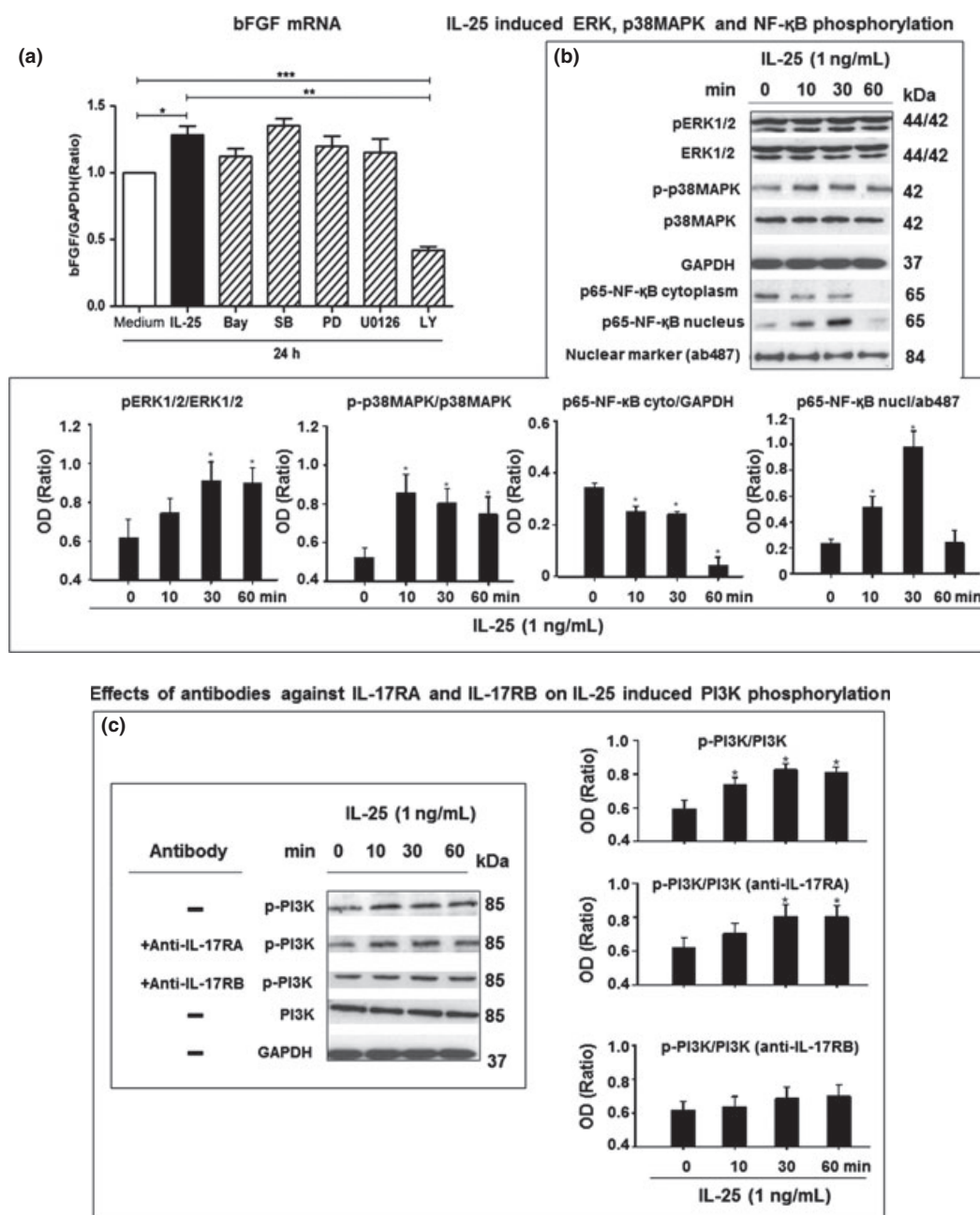


Fig. 6. (a) Real-time PCR analysis of basic fibroblast growth factor mRNA expression in human vascular endothelial cells stimulated (24 h) with medium control or IL-25 (1 ng/mL) with or without the signalling inhibitors Bay 11-7082 (Bay, 2.5 μ M), SB203580 (SB, 5.0 μ M), PD 98059 (5.0 μ M), U0126 (10.0 μ M) and LY 2794002 (LY, 10.0 μ M). Bars show mean $\pm$ SEM ($n = 5$). Student's paired t -test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (b) Western blotting to show IL-25-induced phosphorylation of ERK1/2 (p-ERK1/2), p38MAPK (p-p38MAPK) and nuclear translocation of phosphorylated NF- κ B with expression of unphosphorylated molecules shown for comparison. Total Glyceraldehyde 3-phosphate dehydrogenase and nuclear matrix protein (ab487) were also used as loading controls. Western blotting (c) showing that IL-25 also induces phosphorylation of PI3K (p-PI3K) which was inhibited by anti-IL-17RB, but not anti-IL-17RA antibody. Data are presented as the mean $\pm$ SEM of three independent experiments. Student's paired t -test. * $P < 0.05$.

HUVEC. We employed 5- to 10-fold higher concentrations of both the monoclonal anti-IL-17RA antibody (MAB1771) reported to neutralize 70–90% of IL-17RA-mediated bioactivity at 2 μ g/mL, and the polyclonal anti-IL-17RA antibody (AF177) reported by the

manufacturers to have a ~50% neutralization concentration of 0.1–0.5 μ g/mL. We also tested the effects of the antibodies used in this study on IL-8 expression in HUVEC. Using the same concentrations of antibodies against IL-17RA and IL-17RB, the effects of IL-17 and

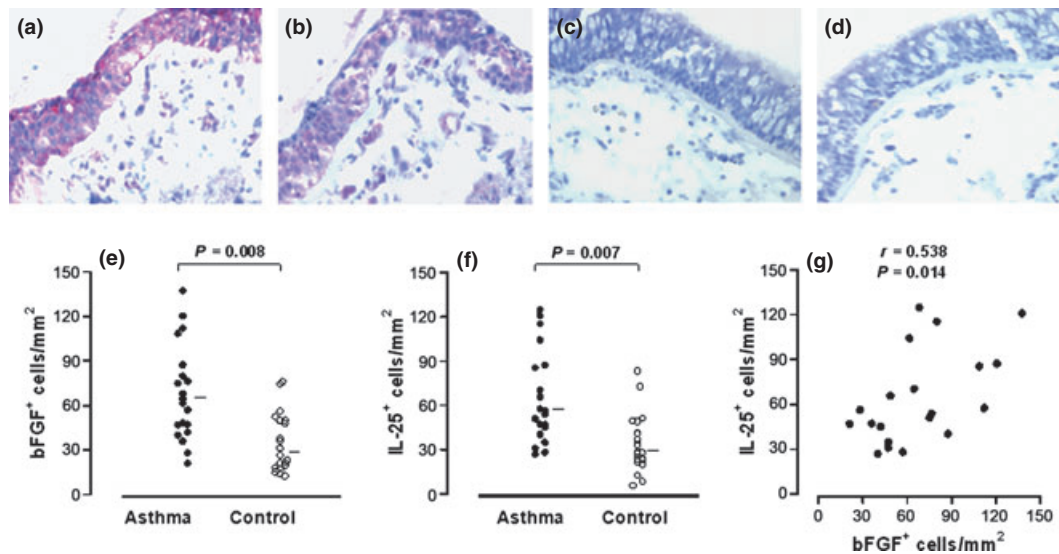


Fig. 7. Immunostaining with anti-basic fibroblast growth factor (a), anti-IL-25 (b) and isotype-matched negative control antibodies (c, d) in sections of bronchial biopsies from asthmatics. Total cells per unit area of entire sections showing bFGF (e) and IL-25 (f) immunoreactivity in bronchial biopsies from asthmatics and controls ($n = 20$ for each group) and the correlation between the numbers of bFGF and IL-25 immunoreactive cells in asthmatics (g). Bars show medians. Mann-Whitney U -test.

IL-25, respectively, on IL-8 expression were effectively abrogated. Furthermore, IL-25-induced angiogenesis was also blocked by IL-17RB, but not by both IL-17RA antibodies. These observations clearly show that IL-25 exerts its functional effects on endothelial cells, at least in terms of inducing bFGF expression, through the IL-17RB receptor.

To investigate the signalling pathways involved in IL-25-induced bFGF expression, we employed a panel of commercially available inhibitors which were also tested in our previous study and did not compromise the viability of the endothelial cells [17]. A very recent report suggested that IL-17RA activates endothelial cells through the p38 MAPK pathway [24]. In this study, inhibition of PI3K by LY294002 completely abrogated IL-25-induced bFGF mRNA expression, which was accompanied by increased phosphorylation of PI3K in HUVEC. In contrast, the p38 MAPK inhibitor SB203580 did not inhibit IL-25-induced bFGF expression even though IL-25-induced detectable phosphorylation of p38MAPK (Fig. 6b). We similarly previously noted that IL-25-induced angiogenesis and expression of VEGF in HUVEC was most clearly inhibited by LY294002. Thus, PI3K signalling plays an essential role in IL-25-induced bFGF, as well as VEGF expression in human endothelial cells. This is in line with other recent studies [29, 30] suggesting that PI3K is a critical signalling pathway for angiogenesis induced in endothelial cells by bFGF and other mediators. Indeed, it has been suggested that PI3K may be a key molecular target in chronic Th2-type inflammatory processes such as asthma [31].

Finally, extrapolating further from our data, we speculate that IL-25 may play a more wide ranging role in tissue remodelling associated with Th2-type inflammation through possible effects on fibrogenesis and smooth muscle hyperplasia as well as angiogenesis, inducing production of bFGF and other remodelling cytokines by a variety of target structural cells.

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