T-helper cell type 2 (Th2) memory T cell-potentiating cytokine IL-25 has the potential to promote angiogenesis in asthma

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IL-25 (IL-17E) is a T-helper cell type 2 (Th2) cytokine best described as a potentiator of Th2 memory responses. Reports of expression of its receptor, IL-25R, on airways structural cells suggest a wider role for IL-25 in remodeling. We hypothesized that IL-25 stimulates local angiogenesis in the asthmatic bronchial mucosa. Immunoreactive IL-25⁺, IL-25R⁺, and CD31⁺ (endothelial) cells in sections of bronchial biopsies from asthmatics and controls were detected by immunohistochemistry. The effect of IL-25 on angiogenesis was examined using an in vitro assay. Real-time PCR was used to detect expression of IL-25R and VEGF mRNA in cultured human vascular endothelial cells (HUVEC), and a cell proliferation kit (WST-8) was used to measure the effect of IL-25 on HUVEC proliferation. Immunostaining showed that IL-25⁺, IL-25R⁺, and CD31⁺/IL-25R⁺ cells were significantly elevated in the bronchial mucosa of asthmatics compared with controls ($P < 0.003$). In asthmatics, the numbers of IL-25⁺ cells correlated inversely with the forced expiratory volume in 1 s ($r = -0.639$; $P = 0.01$). In vitro, HUVEC constitutively expressed IL-25R, which was up-regulated further by TNF- α . IL-25 and TNF- α also increased expression of VEGF and VEGF receptors. IL-25 increased HUVEC proliferation and the number, length, and area of microvessel structures in a concentration-dependent manner in vitro. VEGF blockade, the PI3K-specific inhibitor LY294002, and the MAPK/ERK1/2 (MEK1/2)-specific inhibitor U0126 all markedly attenuated IL-25-induced angiogenesis, and the inhibitors also reduced IL-25-induced proliferation and VEGF expression. Our findings suggest that IL-25 is elevated in asthma and contributes to angiogenesis, at least partly by increasing endothelial cell VEGF/VEGF receptor expression through PI3K/Akt and Erk/MAPK pathways.

Airways remodeling in asthma refers to structural changes, which include increased angiogenesis (1–4). Previous studies suggest that neovascularization and microvascular leakage are prominent in asthmatic airways (1–5). VEGF is one of the most potent proangiogenic factors (6).

IL-25 (IL-17E) is a member of family of six proteins labeled IL-17A–F. IL-17A and IL-17F are expressed by a novel subset of CD4⁺ T-helper (Th) cells and appear to play critical roles in inflammation and autoimmunity, whereas IL-25 is exceptional in promoting Th cell type 2 (Th2) immune responses (7). In mice, exogenous administration of IL-25 (8, 9) or transgenic expression (10, 11) induces asthma-like features. Conversely, anti-IL-25 antibody reduces airways inflammation in animal models of asthma (12, 13). In addition to T cells, lung epithelial cells, mast cells, basophils, and eosinophils may produce IL-25 (14, 15).

The IL-25 receptor (IL-25R) is a 56-kDa single-transmembrane protein. We demonstrated (14) that human Th2 central memory cells selectively up-regulate IL-25R when stimulated with thymic stromal lymphopoietin-activated dendritic cells or with T-cell memory “homeostatic” cytokines such as IL-7 or when triggered by specific antigen. This up-regulation resulted in enhanced sensitivity of the cells to IL-25 that was associated with IL-4-independent, sustained expression of GATA3, c-MAF, and

JunB. This finding and the further observation (16) that IL-25 activates eosinophils to produce a range of asthma-relevant mediators suggest that IL-25 may play a pivotal role in maintaining Th2 central memory and sustaining asthmatic inflammation, whereas its production by mast cells and eosinophils suggests the possibility of a positive feedback amplifying loop.

IL-25R also is expressed on human eosinophils, monocytes, airways smooth muscle cells, and fibroblasts (16–19), raising the possibility that IL-25 also may be involved in causing structural changes in the airways that characterize asthma. However, its possible role in angiogenesis has never been explored. Our preliminary data (20) showed that immunoreactive IL-25R colocalized with CD31⁺ blood vessel endothelial cells. Thus, for the present study, we hypothesized that IL-25 plays a role in angiogenesis in asthma. Our aim was to measure expression of IL-25 and its receptor in the bronchial mucosa of asthmatics and controls (in controls, particularly on vascular endothelial cells) and to characterize the phenomenon and mechanisms of IL-25-induced angiogenesis.

Results

Clinical Data. In this study we compared bronchial biopsies from 15 asthmatics and 15 controls. Clinical and demographic details of the study subjects are summarized in Table S1.

IL-25 and IL-25R Immunoreactive Cells and CD31⁺/IL-25R⁺ Microvessels in Vivo. The median number of cells showing immunoreactivity for IL-25 and IL-25R was significantly higher in the bronchial mucosa of the asthmatics than in controls (Fig. 1 *A*, *B*, *E*, and *F*). In the asthmatics, the number of IL-25⁺ but not IL-25R⁺ immunoreactive cells correlated inversely with the forced expiratory volume in 1 s ($r = -0.639$; $P = 0.01$). The total number of IL-25⁺ immunoreactive cells correlated positively with the number of IL-25R⁺ immunoreactive cells ($r = 0.522$, $P = 0.046$). IL-25⁺ immunoreactive cells were located in both the epithelium and the submucosa, whereas IL-25R⁺ immunoreactive cells were located almost exclusively in the submucosa (Fig. 1 *A* and *B*). The number of CD31⁺ and CD31⁺/IL-25R⁺ vascular structures

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Fig. 1. (A–D) Immunoreactivity for IL-25 (A), IL-25R (B), CD31 (D), and negative control staining for IL-25 (C) in sections of the asthmatic bronchial mucosa. (E–H) Total cells per unit area of entire sections showing IL-25 (E) and IL-25R (F) immunoreactivity and vascular structures showing CD31 (G) and CD31⁺/IL-25R⁺ (H) immunoreactivity in bronchial biopsies from asthmatics and controls ($n = 15$ for each group). Bars indicate medians. Mann–Whitney U test.

also was significantly higher in asthmatics than in controls (Fig. 1 *G* and *H*).

IL-25R Expression in Human Vascular Endothelial Cells in Vitro. In human vascular endothelial cells (HUVEC), constitutively expressed IL-25R mRNA, protein, and immunoreactivity were increased in the presence of TNF- α (Fig. 2 *B*, *E*, *G*, and *H*). Interestingly, IL-25 also up-regulated mRNA and protein encoding its own receptor in a concentration-dependent fashion (Fig. 2 *C*, *F*, *G*, and *I*). Western blotting showed that the expression of IL-25R protein was maximal ≈ 48 h after stimulation (Fig. 2*G*).

IL-25-Induced Angiogenesis and VEGF Expression by Human Endothelial Cells in Vitro. In an in vitro angiogenesis assay, IL-25 induced lengthening and branching of microvascular tubules by HUVEC in a concentration-dependent fashion as compared with VEGF, which was used as a positive control (Fig. 3 *A–H*). IL-25 also induced proliferation and expression of VEGF mRNA by HUVEC (Fig. 3 *I* and *K*) and human pulmonary microvascular endothelial cells (HPMEC) (Fig. 3 *J* and *L*). These effects were not inhibited by blockade of the IL-17A receptor (IL-17RA) (Fig. 3 *I–L*).

IL-25 Up-Regulated Expression of VEGF Receptors in HUVEC in Vitro. IL-25 also significantly up-regulated expression of mRNA encoding VEGF receptors 1 and 2 (VEGFR1 and VEGFR2) in HUVEC in a time- and concentration-dependent fashion (Fig. 4 *A* and *B*). Expression of VEGFR1 and -2 proteins was correspondingly increased with a peak at ≈ 48 h (Fig. 4 *C*, *D*, and *E*).

Effects of Signaling Inhibitors on Angiogenesis and VEGF Protein Production. The PI3K inhibitor LY 294002 and the MEK inhibitor U0126, but not the p38 MAPK inhibitor SB 203580, completely attenuated IL-25-induced formation of new microvascular structures by HUVEC in vitro (Fig. 5 *A–C*). A similar picture was observed with VEGF-induced angiogenesis. Cellular viability remained $>95\%$ throughout all cultures. Anti-VEGF neutralizing monoclonal antibody attenuated the angiogenic effect of IL-25 by up to 70% and also reduced spontaneous angiogenesis but did not completely ablate the effects of IL-25. Preneutralization of IL-25 with anti-IL-25 antibody completely blocked IL-25-induced angiogenesis. Furthermore, ELISA showed that anti-IL-25 ablated IL-25-induced VEGF production in the angiogenesis assay, and LY294002 and U0126 further reduced it below baseline (Fig. 5*D*).

Effects of Signaling Inhibitors on Proliferation and VEGF mRNA Expression by HUVEC. HUVEC were coincubated with IL-25 and a range of signaling inhibitors for 24 h for quantitative PCR and for 48 h for the proliferation assay. The proliferation assay

Fig. 2. (A–D) Immunoreactive IL-25R on chamber-cultured HUVEC following culture for 48 h in (A) medium alone; (B) TNF- α (10 ng/mL); (C) IL-25 (10 ng/mL); and (D) as in B but with substitution of the anti-IL-25R antibody with an isotype control (40 $\times$ magnification). (E and F) Real-time PCR showing time courses and concentration response relationships of (E) TNF- α - and (F) IL-25-induced IL-25R mRNA expression. (G–I) Western blots showing (G) time course of TNF- α - (10 ng/mL) and IL-25- (1 ng/mL) induced IL-25R protein expression, (H) concentration-dependent TNF- α -induced, and (I) IL-25-induced IL-25R expression at 48 h. Bars represent mean $\pm$ SEM ($n = 4$). * $P < 0.05$. Mann–Whitney U test.

them to be comparable. Spontaneous and IL-25-induced angiogenesis was extensively but not completely inhibited by VEGF blockade, suggesting that IL-25 acts principally by increasing

VEGF production. This incomplete inhibition was verified by VEGF ELISA, although a possible direct effect of IL-25 on endothelial cells or the possibility that IL-25 induces the production of angiogenetic mediators other than VEGF cannot be excluded. We then verified that IL-25 increases the expression not only of VEGF but also its two principal receptors, VEGFR1 and -2, in vascular endothelial cells, again suggesting that IL-25 increases the potential capacity of these cells to respond to VEGF. The bell-shaped concentration-response curves for IL-25 in inducing expression of IL-25R, VEGF, and VEGF receptors by HUVEC (Figs. 2, 3, and 4) are compatible with the hypothesis that higher concentrations of IL-25 induce IL-25R internalization, as observed with some cytokines and chemokines and their receptors (e.g., as IL-5 and eotaxin) (24, 25).

Recent reports (26, 27) have suggested that endothelial cells also express IL-17RA and that certain activities of IL-25 can be exerted through this receptor. Therefore we examined the effects of an anti-IL-17RA blocking antibody. Anti-IL-17RA did not attenuate endothelial cell proliferation or VEGF expression (Fig. 3).

The signaling pathways used by IL-25 when acting on its receptor IL-25R have not been well defined and may vary by cell type and species. Previous studies (16, 28) suggest a role for p38 MAPK, JNK, and NF- κ B in T cells and eosinophils in humans and TNF receptor-associated factor 6 and the adaptor protein connection to IK K-complex and SAPK (CIKS)/Akt in mice (29–31). To investigate the signaling pathways involved in IL-25-induced VEGF expression, we used a panel of commercially available inhibitors. Inhibition of PI3K or MEK1/2 with the pharmacological inhibitors LY294002/SH-6 and U0126, respectively, completely attenuated IL-25-induced VEGF mRNA expression and proliferation in endothelial cells. Inhibition of Akt (SH-6), MEK1 alone (with PD98059), or NF- κ B (with Bay 11–7082) showed measurable but lesser effects, whereas the Stat3 inhibitor WP-1066, the P38 MAPK inhibitor SB203580, and the JNK inhibitor SP600125 were ineffective. Taking together these and previously published data, we

have summarized in Fig. S2 the signaling pathways that, to date, have and have not been implicated in IL-25 signaling. For reasons of practicality and cost, we did not perform full concentration–response curves with all the signaling inhibitors but used concentrations that were physiologically relevant but did not compromise the viability of the endothelial cells. The effects of these inhibitors on VEGF production and VEGF-induced vascular formation in the present study closely mirrored their activities in inhibiting IL-25-enhanced angiogenesis. These data are consistent with a role for PI3K/Akt and MEK/Erk signaling in IL-25-induced angiogenesis, although they do not allow discrimination between effects on IL-25 signaling inducing VEGF and VEGFR expression or VEGF signaling inducing angiogenesis, or both. Our data suggest that blocking PI3K, MEK, and NF- κ B reduces IL-25-induced proliferation and VEGF expression by endothelial cells, two critical factors in the regulation of angiogenesis.

Other recent studies also suggest that PI3K plays a critical role in cytokine production and angiogenesis by vascular endothelial cells (32–34); indeed, it has been suggested more generally that PI3K may be a key molecular target for the therapy of asthma (35). In the present study, we were unable to examine inhibition of Stat6 signaling because of the lack of commercially available specific inhibitors. However, we did observe that IL-25 enhanced phosphorylation of signal transducer and activator of transcription 6 (Stat6) but not of Stat3, suggesting that Stat6 also might mediate IL-25-induced angiogenesis through its effects on the proliferation of endothelial cells and expression of VEGF. Some caution must be exercised when interpreting our data showing an apparent lack of effect of the p38 MAPK inhibitor SB203580 and the JNK inhibitor SP600125 on IL-25-induced VEGF mRNA expression and angiogenesis, because, as mentioned, one may be observing the net effect of influences on IL-25- and VEGF-mediated signaling. Issbrücker and colleagues (36) previously reported that SB203580 enhanced VEGF-induced angiogenesis by endothelial cells.

Increased angiogenesis is a well-documented feature of airways remodeling in asthma. We (37) and others (38) have shown elevated expression of angiogenic mediators, including VEGF, fibroblast growth factor, platelet-derived growth factor, and angiogenin in the airways tissue and lining fluid of asthmatics, and the expression of these mediators can be correlated positively with disease severity and negatively with airways obstruction. VEGF appears to be key among these mediators, and IL-25 may make a significant contribution to its expression. Because airways fibroblasts and smooth muscle cells also express IL-25R (17, 19), we speculate that IL-25 may play an even wider role in airways remodeling through its effects on fibrogenesis and smooth muscle hyperplasia. For the moment, our data extend the knowledge of the manifold possible roles for IL-25 in asthma pathogenesis and point to it and its putative signaling molecules PI3K/Akt and MEK1/2/Erk as candidate molecular targets for novel therapeutic approaches.

Methods

Study Patients. The study was approved by the Guy's Hospital Ethics Committee and was carried out in our clinical research facility. Each subject provided written, informed consent. Endobronchial biopsies were obtained from 15 asthmatics and 15 normal controls. Details are given in *SI Methods* and Table S1.

Fiberoptic Bronchoscopy. Fiberoptic bronchoscopy was performed as previously described (39, 40). Details are given in *SI Methods*.

Immunohistochemistry. To identify cells expressing IL-25, IL-25R, and CD31, we used the primary antibodies mouse anti-human IL-17E, MAB 1258 (1:20; R&D Systems), mouse anti-human IL-25R (1:50; LB-2044; Autogene Bioscience), and mouse anti-human CD31 (1:30; M0823; Dako Ltd.) and well-established protocols that we have described previously (39, 40). Positively staining cells were detected with Fast Red (Sigma) (39, 40). Further details are given in *SI Methods*. The mean $\pm$ SD of the entire cross-sectional areas of the biopsy sections examined in the asthma and control groups were 2.6 ± 0.3 mm² and 2.8 ± 0.4 mm², respectively, with within-group coefficients of variation ranging from 8.79–10.2%. The between-observer coefficients of variation from duplicate counts for all markers tested for two groups ranged from 3.2–5.1%.

HUVEC Culture. All reagents and HUVEC were purchased from PromoCell unless otherwise specified. Cells before passage four were used for experiments. Briefly, cells (0.5×10^6 per well) were seeded in six-well plates (Nunc A/S) precoated with gelatin (0.5%; Sigma-Aldrich) in complete medium (endothelial cell media kit; PromoCell) containing basal medium, 0.4% endothelial cell growth supplement, 2% FCS, 0.1 ng/mL EGF, 1 ng/mL basic FGF, 100 U/mL penicillin, and 100 μ g/mL streptomycin and grown to $\approx$ 80% confluence in a 5% CO₂ incubator at 37 °C. The cells then were exposed to stimuli including recombinant human TNF- α (R&D) and IL-25 (R&D Systems). To identify IL-25R immunoreactivity on HUVEC, some HUVEC were seeded and cultured to $\approx$ 80% confluence in chamber slides (Nalge Nunc International) for 48 h with or without recombinant human TNF- α . The effects of a number of signaling inhibitors were tested in this system; a full description is given in *SI Methods*. Cells were harvested for further investigation at different time points, as described in *Results*. Cellular viability was assessed by Trypan blue exclusion.

RNA Extraction and Real-Time PCR. Total cellular RNA was extracted from HUVEC using the RNeasy Mini kit (QIAGEN). The RNA was reverse transcribed and subjected to quantitative real-time PCR analysis (further details are given in *SI Methods*). Real-time PCR primers were 5'-AACAGGCGTCCCTTCCCTCTGGA-3' and 5'-TTCTTGATCCTTCTGTGCTCCAC-3' for human IL-25R (IL-17BR); 5'-TACTCCACCATGCCAAGTG-3' and 5'-GATGATTCTGCCCTCCTCTT-3' for human VEGF; 5'-TCACTGCCACTCTAATTGTCAATGT-3' and 5'-AAACGATGACACGGCCTTTT-3' for human VEGF receptor 1 (VEGFR1); and 5'-CAAGACAGGAAGACCAAGAAAAGAC-3' and 5'-GGTGCCACACGCTCTAGGA-3' for human VEGF receptor 2 (VEGFR2).

Western Blotting. Western blotting was performed as previously described (38). HUVEC were cultured with or without stimuli for 24, 48, or 72 h in a 5% CO₂ incubator at 37 °C. Cells were washed with ice-cold PBS and then lysed in 50 μ L 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) containing a protease inhibitor mixture (Roche) and 10 mM sodium orthovanadate (Sigma). Further details are given in *SI Methods*. The receptor proteins were detected by incubation with antibodies against human IL-25R (1:2,000; LifeSpan Biosciences) (17), VEGFR1 (1:4,000; Abcam plc) and VEGFR2 (1:1,000; Cell Signaling Technology, Inc.) (41) for 16 h. After washing, the membrane was incubated with HRP-conjugated goat anti-mouse for IL-25R detection (1:3,000; Dako) or HRP-conjugated goat anti-rabbit for VEGFR detection (1:10,000; Abcam) in blocking buffer for 45 min at room temperature and subsequently were developed with Western blotting detection reagents (Amersham) according to the manufacturer's instructions.

In separate experiments, cells were incubated with or without IL-25 (R&D Systems) to detect IL-25-induced phosphorylation of signaling molecules (42). Antibodies were purchased from Cell Signaling Technology, Inc. and are listed, along with the dilutions used, in *SI Methods*. Total GAPDH and nuclear matrix protein p84 were detected by monoclonal antibodies (GAPDH: WH0002597M1; 1:5,000; Sigma; nuclear matrix protein p84: ab487; 1:1,000; Abcam plc) as loading controls for cytoplasmic and nuclear proteins, respectively (43, 44).

HUVEC Proliferation Assay. HUVEC proliferation was measured using a commercial kit (WST-8, Cayman Chemicals) (45). Briefly, 1×10^4 cells in 100 μ L of medium were plated on a 96-well microplate. After 24 h of incubation, aliquots of 100 μ L medium with several concentrations of recombinant human IL-25 were added. After incubation for 24 or 48 h, the medium was replaced with new medium containing 10% WST-8. After 1 h of incubation, the formation of formazan was determined photometrically at 450 nm with a microplate reader. Recombinant human VEGF (10 ng/mL) was used as a positive control. All analyses were performed on sets of four wells. The results are expressed as percentages of medium control values. To explore the generality of these properties of endothelial cells, similar experiments were performed on microvascular endothelial cells of pulmonary origin (PromoCell GmbH). To investigate whether IL-25 binds to the alternative receptor IL-17RA (26, 27), cells were precultured with 4 μ g/mL anti-IL-17RA monoclonal neutralizing antibody (MAB177; R&D Systems) for 30 min before recombinant human IL-25 (10 ng/mL) was added for a further 24-h incubation. After culturing, cells were harvested for proliferation assays and real-time PCR to measure VEGF mRNA expression, as described.

In Vitro Angiogenesis Assay. The effects of IL-25 on angiogenesis were investigated using an in vitro angiogenesis assay (34, 35) based on coculture of HUVEC for 11 d over a monolayer of irradiated fibroblasts (AngioKit; TCS

CellWorks). Further details are given in *SI Methods*. To evaluate whether VEGF is involved in IL-25-induced angiogenesis, a monoclonal neutralizing antibody against human VEGF (1 $\mu\text{g/mL}$) (R&D Systems) was added to some wells on day 0 and replenished on days 4, 7, and 9. Preincubation of IL-25 with anti-IL-25 antibody (1 $\mu\text{g/mL}$) (R&D Systems) also was used to neutralize IL-25. Additionally, IL-25 signaling pathways inducing angiogenesis were probed by adding specific inhibitors including SB203580, U0126, and LY294002 (10.0 $\mu\text{mol/L}$ for the first 3 d, followed by 2.0 $\mu\text{mol/L}$ until the end of the 11-d culture period) (31, 34).

ELISA for VEGF. At day 9, supernatants were collected from the angiogenesis assays. ELISA kits were used to measure human VEGF according to the manufacturer's instructions (R&D Systems). The limit of detection was 63 pg/mL.

Statistical Analysis. Data were analyzed using the Minitab Release 7 statistical package (Minitab Inc.). The Mann–Whitney *U* test was used for analyzing all data, and $P < 0.05$ was considered significant.

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