Supporting Information

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SI Methods

Study Patients. Asthmatics had a clear history of relevant symptoms, documented reversible (≥12%) airways obstruction, and/or histamine PC_{20} <8 mg/mL measured within 2 wk before biopsy. None had ever smoked, and there was no history of other respiratory disease. All subjects were clinically free of respiratory infection and systemic glucocorticoid therapy for at least 1 mo before the study. Atopy was defined by standard skin-prick testing. Normal control subjects were healthy, life-long nonsmoking volunteers who had no history of lung disease.

Fiberoptic Bronchoscopy. All subjects were premedicated with 2.5 mg nebulized salbutamol and 0.6 mg i.v. atropine. Sedation was achieved with i.v. midazolam (1–10 mg) or alfentanil (1–500 μg). Biopsies were taken from right, middle, and lower lobe bronchi using Olympus alligator forceps (model FB15C). All subjects were given a further 2.5 mg of nebulized salbutamol immediately after the procedure. Bronchoscopy was well tolerated by all subjects, and safety was monitored prospectively.

Immunohistochemistry. Omission or substitution of the primary antibody with an irrelevant antibody of the same species and isotype was used as a negative control. The slides were observed in blinded fashion on an Olympus BX40 microscope connected with a Zeiss Vision KS300 imaging system (Carl Zeiss), and total numbers of IL-25^+ and IL-$25R^+$ cells and the number of $CD31^+$ blood vessels were counted in entire biopsy sections. The data were expressed as the total number of cells expressing immunoreactivity for IL-25 and IL-25R per unit area of bronchial submucosa. $CD31^+$ endothelial cells coexpressing IL-25R were identified by double sequential technique [alkaline phosphatase anti-alkaline phosphatase (APAAP) for CD31, avidin-biotin-peroxidase complex (ABC) for IL-25R].

Culture of Human Vascular Endothelial Cells. To test the effects of signaling inhibitors, a number of reagents were used, including SB203580 (p38 MAPK inhibitor) (Promega Ltd.), U0126 [MAPK/ERK1 and -2 (MEK1 and MEK2) inhibitor] (Calbiochem Corp.), LY294002 (PI3K inhibitor) (Merck Chemicals Ltd.), SH-6 (Akt serine/threonine kinase inhibitor) (Merck), PD98059 (MEK1 inhibitor) (Cayman Chemical Co.), SP600125 (JNK inhibitor) (Cayman Chemical Co.), WP-1066 (Stat3 inhibitor) (Sigma), and Bay11-7082 (NF-κB inhibitor) (Cayman Chemical Co.).

RNA Extraction and Real-Time PCR. Two micrograms of RNA were DNase treated and then reverse transcribed according to kit instructions. Quantitative real-time PCR was performed on a 7500 Real-Time PCR System (Applied Biosystems). mRNA copy numbers were calculated for each sample by using the cycle threshold (Ct) value. rRNA from the *18s* housekeeping gene was amplified in parallel with tested genes. The number of amplification steps required to reach an arbitrary Ct intensity was computed. The relative gene expression was represented by $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta C_{Target\ gene} - \Delta C_{18s}$.

Western Blotting. Cell debris was removed by centrifugation at 15,000 × *g* for 20 min at 4 °C. The supernatants were boiled in 6× Laemmli sample buffer (Bio-Rad Laboratories Ltd.) for 6 min. The protein contents in the supernatants were determined using the bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific). Equal amounts of protein (20 μg protein per lane) were subjected to electrophoresis, transferred to nitrocellulose membranes, and then incubated in blocking buffer [0.05% Tween-20 (pH 7.4), 5% fat-free milk] for 1 h at room temperature. Antibodies used for testing IL-25–induced phosphorylation included total ERK1/2 (137F5) and phosphorylated ERK1/2 (p-ERK1/2) (197G2) (1:2,000); total PI3K p85 (19H8), and p-PI3K p85/p55 (Tyr458/Tyr199) (1:1,000); total Akt (C67E7) and p-Akt (Ser473) (1:1,000); total Stat3 (9132) and p-Stat3 (Tyr705) (9131) (1:1,000); total Stat6 (9362) and p-Stat6 (Try641) (9361) (1:1,000); total IκBα (4812); and p65 NFκB (Ser536) (3036) (1:1,000).

In Vitro Angiogenesis Assay. Cultures were incubated at 37 °C in a humidified atmosphere with 5% CO_2. Culture medium with recombinant human IL-25 (1, 10 ng/mL) (R&D Systems) was replenished on days 4, 7, and 9. Culture medium alone and recombinant human VEGFA (10 ng/mL) (R&D Systems) served as negative and positive assay controls, respectively. On day 11 cultured cells were fixed, and vascular structures were visualized by labeling with mouse anti-CD31 according to the manufacturer's instructions. Multiple photomicrographs (4× objective) (four separate fields per well) were taken at clock points 12, 3, 6, and 9, and angiogenesis in each field of view was quantified using image analysis software (AngioSys; TCS CellWorks). The analysis software segmented the images using a gray-level threshold tool to select CD31-labeled cells. The resultant binary images were skeletonized, and branch points were removed to determine the total lengths of individual tubules. Branch points were counted, and the total area of CD31 labeling was determined from the original binary images, permitting overall numbers of vascular junctions, tubules, and tubule length to be determined.

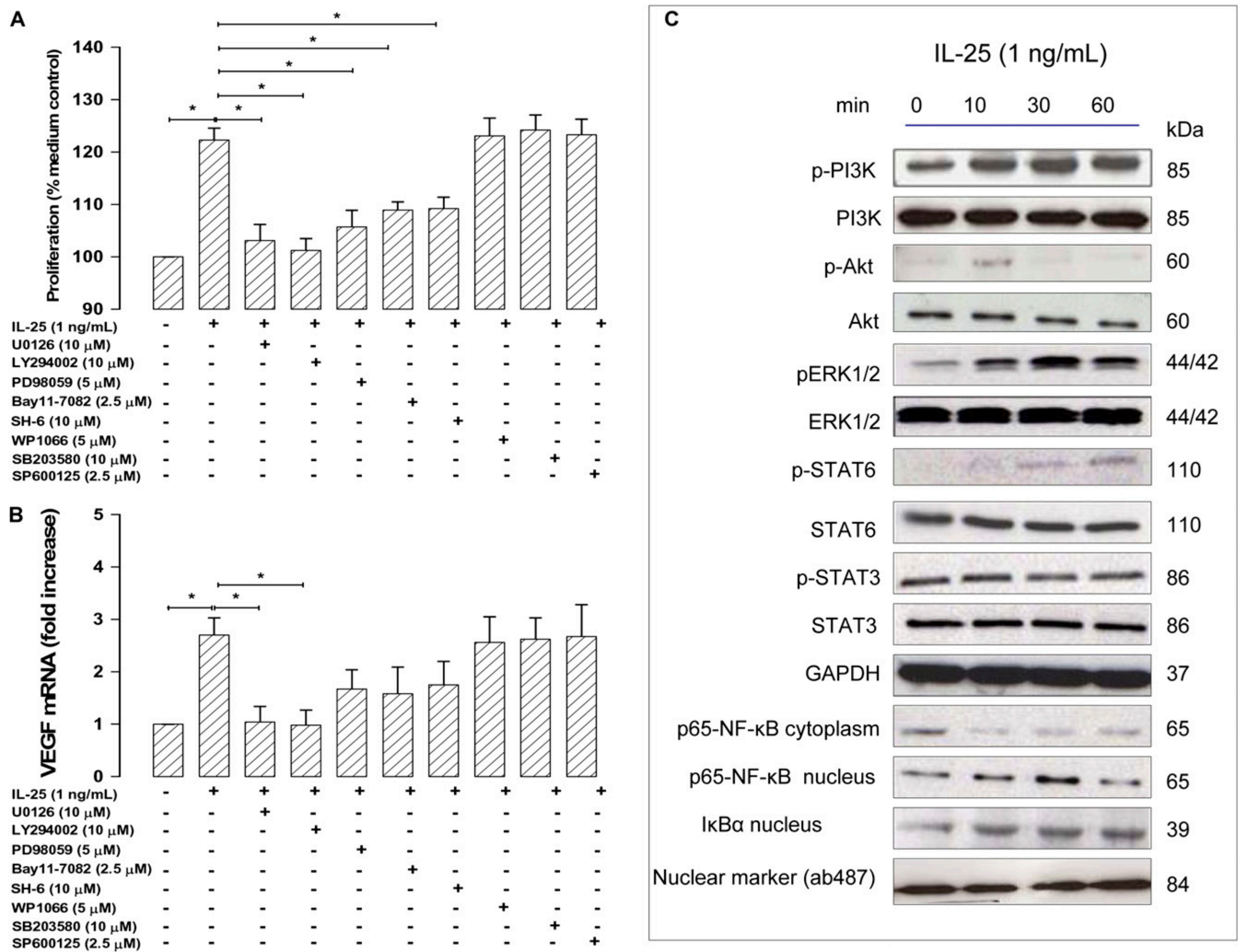

Fig. S1. Effects of IL-25 on proliferation of human vascular endothelial cells. VEGF mRNA expression and signaling molecule phosphorylation. Inhibitors of PI3K (LY294002), Akt (SH-6), MEK1/2 (U0126), MEK1 (PD98059), and NF-κB (Bay11-7082), but not of Stat3 (WP-1066), JNK (SP600125), or p38 (SB203580), significantly attenuated IL-25–induced proliferation (*A*) and VEGF mRNA expression (*B*). $^*P < 0.05$. Mann–Whitney *U* test. (*C*) Western blot shows IL-25–induced phosphorylation of PI3K, Akt, Erk1/2, and Stat6 (but not Stat3) and nuclear translocation of phosphorylated NF-κB. Expression of unphosphorylated molecules is shown for comparison. Total GAPDH and nuclear matrix protein (ab487) also were used as loading controls.

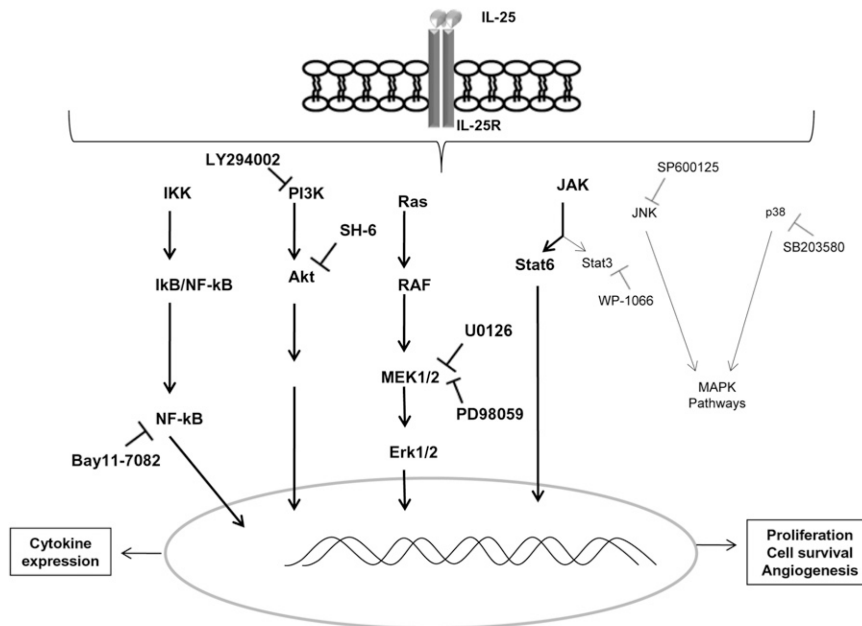

Fig. S2. Proposed signaling pathways for IL-25 in endothelial cells. IL-25 binding with its receptor causes proliferation, angiogenesis, cytokine (including VEGF) expression, and phosphorylation of PI3K, Akt, Erk1/2, NF-κB, and Stat6 in endothelial cells. Inhibitors of PI3K/Akt, MEK1/2, NF-κB, and Stat6 (shown in bold), but not of JNK, p38, or Stat3, diminish the effects of IL-25.

Table S1. Clinical and demographic data for study subjects

Subjects	Age in y	Forced expiratory volume in 1 s (% predicted)*	Therapy (beclometasone dosage equivalent in μg/d)
Asthma (n = 15)	44 (27–57)	77.3 (43.2–109.6)	4 LABA, 6 IG 300 (200–1,000)
Control (n = 15)	28 (23–60)	122.0 (89–134)	

All patients were taking short-acting β2-agonist as required. Six of 15 asthmatics and 9/15 controls were female. All asthmatics and 6/15 controls were atopic. IG, inhaled glucocorticoid; LABA, long-acting β2-agonist.

*Data are expressed as the median (range). $P < 0.001$ vs. control (Mann–Whitney U test).