IL-17 Enhances the Net Angiogenic Activity and In Vivo Growth of Human Non-Small Cell Lung Cancer in SCID Mice through Promoting CXCR-2-Dependent Angiogenesis[1]

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In this study, we examined the biological action of IL-17 on human non-small cell lung cancer (NSCLC). Although IL-17 had no direct effect on the in vitro growth rate of NSCLC, IL-17 selectively augmented the secretion of an array of angiogenic CXC chemokines, including CXCL1, CXCL5, CXCL6, and CXCL8 but not angiostatic chemokines, by three different NSCLC lines. Endothelial cell chemotactic activity (as a measure of net angiogenic potential) was increased in response to conditioned medium from NSCLC stimulated with IL-17 compared with those from unstimulated NSCLC. Enhanced chemotactic activity was suppressed by neutralizing mAb(s) to CXCL1, CXCL5, and CXCL8 or to CXCR-2 but not to vascular endothelial growth factor-A. Transfection with IL-17 into NSCLC had no effect on the in vitro growth, whereas IL-17 transfectants grew more rapidly compared with controls when transplanted in SCID mice. This IL-17-elicited enhancement of NSCLC growth was associated with increased tumor vascularity. Moreover, treatment with anti-mouse CXCR-2-neutralizing Ab significantly attenuated the growth of both neomycin phosphotransferase gene-transfected and IL-17-transfected NSCLC tumors in SCID mice. A potential role for IL-17 in modulation of the human NSCLC phenotype was supported by the findings that, in primary NSCLC tissues, IL-17 expression was frequently detected in accumulating and infiltrating inflammatory cells and that high levels of IL-17 expression were associated with increased tumor vascularity. These results demonstrate that IL-17 increases the net angiogenic activity and in vivo growth of NSCLC via promoting CXCR-2-dependent angiogenesis and suggest that targeting CXCR-2 signaling may be a novel promising strategy to treat patients with NSCLC. ***The Journal of Immunology,*** **2005, 175: 6177–6189.**

Interleukin 17, initially termed CTLA-8 (1), is produced mainly by activated CD4 T cells (2) and has sequence homology with the open reading frame 13 of the T lymphotropic *Herpesvirus saimiri* (2). Several recently discovered homologous proteins with similar or different biological profiles including IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F form a novel cytokine family (3–5). IL-17 has pleiotropic biological activities including induction of IL-6, CXCL8/IL-8 and vascular endothelial growth factor-A (VEGF-A),[3] as well as enhancement of ICAM-1 expression (2–11). In addition, IL-17 induces the secretion of TNF-α and IL-1β by activated macrophages (12). IL-17R is a type 1 transmembrane protein with an extraordinarily long intracellular domain (2, 13). Although the expression of IL-17 mRNA is restricted to activated T cells, the expression of IL-17R mRNA has been detected in virtually all cells and tissues (2, 13).

In its role as a proinflammatory cytokine, IL-17 levels have been found to significantly increase in rheumatoid arthritis synovium, asthmatic airways, during allograft rejection, and in other chronic inflammatory diseases, including multiple sclerosis and psoriasis (14–18). IL-17 has been also implicated in the tumors. Ciree et al. (19) reported that cutaneous T cell lymphomas spontaneously secrete IL-17, which is associated with infiltration of polymorphonuclear neutrophils into inflamed tissues. Kato et al. (20) reported that a considerable proportion of ovarian carcinomas naturally express IL-17 and its expression significantly correlates with the increased vascularity. However, until now, the possible role for IL-17 in non-small cell lung cancer (NSCLC) has not been elucidated.

The salient feature of solid tumor growth such as NSCLC is the strict dependence on the sustained new vessel growth (21). Hanahan and Folkman (22) proposed that quiescent endothelium exists in a net balance of angiostatic factors over angiogenic ones, thereby maintaining homeostasis. Conversely, tumors themselves, interacting stromal cells and/or responding inflammatory cells, may cause an imbalance to increase the secretion of angiogenic inducers or decrease the production or effect of angiogenic suppressors. Although there are a wide variety of putative mediators of angiogenesis, certain tumors have been found to produce factors

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[3] Abbreviations used in this paper: VEGF-A, vascular endothelial growth factor-A; NSCLC, non-small cell lung cancer; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; CM, conditioned medium; NEAA, nonessential amino acid; LMVEC, lung microvascular endothelial cell; HPF, high-powered field; PDGF, platelet-derived growth factor; Neo, neomycin phosphotransferase gene; DAB, 3,3′-diaminobenzidine; ELR, glutamic acid-leucine-arginine; aFGF, acidic FGF.

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that are directly angiogenic while others may depend upon vascularization induced by products of infiltrating inflammatory cells (23).

NSCLC cells have been reported to produce a wide variety of angiogenic factors, including CXCL1/growth related oncogene-α, CXCL5/epithelial cell-derived neutrophil-activating protein-78, CXCL6/granulocyte chemotactic protein-2, CXCL8/IL-8, VEGF-A, fibroblast growth factors (FGFs), hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF)-BB, TGF-α, CXCL12/stromal derived factor-1α, angiopoietin-2, and PGE_2 over the course of their growth (24–32). Especially, multiple studies have established that angiogenic CXC chemokines are critically involved in the angiogenic activity of NSCLC (22, 33, 34). Arenberg et al. (33) reported that inhibition of the action of CXCL8 markedly suppressed the in vivo growth of NSCLC in SCID mice model. Arenberg et al. (34) also showed that neutralization of CXCL5 in vivo significantly suppressed the growth of NSCLC. In contrast, tumor-derived CXCL10/IFN-inducible protein-10 is an important endogenous angiostatic factor in NSCLC, which negatively regulates tumor angiogenesis and growth (35).

IL-17 induces CXCL8 production from various cell types and CXCL8 is involved in the angiogenic activity of NSCLC. Therefore, we hypothesized that IL-17 might enhance the net angiogenic activity and promote the tumorigenicity of NSCLC. To address this issue, we examined the biological effect of IL-17 on NSCLC and found that IL-17 selectively enhanced the production of an array of angiogenic CXC chemokines, including CXCL1, CXCL5, CXCL6, and CXCL8 but not angiostatic chemokines, from NSCLC lines in vitro. Therefore, these characteristic biologic activities of IL-17 prompted us to further investigate the role for this cytokine in in vivo biologic action of NSCLC. In this study, we show that IL-17 significantly enhances the net angiogenic activity and in vivo growth of NSCLC through promoting CXCR-2-dependent angiogenesis.

Materials and Methods

Mice and reagents

Male SCID mice, 5–7 wk of age, were obtained from Charles River Laboratories. Human IL-17 cDNA was supplied by Schering-Plough. Recombinant human IL-17 and IL-23 proteins and anti-human IL-17, IL-17R, CXCL1, CXCL5, CXCL8, VEGF-A, HGF, CXCR-1, and CXCR-2 mAbs were purchased from R&D Systems. Anti-human IFN-γ mAb was supplied by Mabtech. Goat anti-human IL-17R polyclonal Ab was from Santa Cruz Biotechnology. Rabbit anti-mouse CXCR-2-neutralizing polyclonal Ab was supplied by Dr. K. Matsushima (University of Tokyo, Tokyo, Japan).

Human NSCLC cell lines, cell cultures, and conditioned medium (CM)

Human NSCLC lines Sq-19 (squamous cell carcinoma), A549 (alveolar cell carcinoma), and LK-87 (adenocarcinoma) were supplied from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer (Tohoku University). These cells were maintained in RPMI 1640 with 10% FCS, 2 mM L-glutamine, 0.1 mM nonessential amino acid (NEAA), 100 IU/ml penicillin, and 100 μg/ml streptomycin (all from Invitrogen Life Technologies). Human lung microvascular endothelial cells (LMVECs) were purchased from Cambrex and maintained in HuMedia-EB2 with 10 ng/ml epidermal growth factor, 1 μg/ml hydrocortisone, 5 ng/ml basic FGF, 10 μg/ml heparin, 39.3 μg/ml dibutyryl cAMP, 50 μg/ml gentamicin, 50 ng/ml amphotericin B, and 5% FCS (all from Kurabo). CM was generated as follows. Cells (1×10^5/ml) were cultured in HuMedia-EB2 containing 3% FCS with or without 50 ng/ml IL-17 for 60 h. Cell-free supernatants were collected and stored at −70°C until use.

Clinical materials

Tumor tissues ($n = 77$) were obtained from patients with NSCLC, who underwent complete resection at surgery without any preoperative therapy.

Flow cytometry

Cells (1×10^6), washed twice with PBS containing 2% FCS and 0.1% NaN_3, were incubated with mouse anti-human IL-17R mAb or with irrelevant normal mouse IgG1 (R&D Systems) at 4°C for 60 min. After washing, the cells were incubated with PE-conjugated goat anti-mouse IgG1 Ab (Caltag Laboratories) at 4°C for 30 min. The cells were analyzed with FACSCalibur (BD Biosciences).

MTT assay

Cells (1×10^3) were seeded into 96-well flat-bottom plates and cultured in RPMI 1640 containing 3% FCS, 2 mM L-glutamine, 0.1 mM NEAA, 100 IU/ml penicillin, and 100 μg/ml streptomycin with or without 0.1–1000 ng/ml human IL-17. On day 5 or 7, cells were washed with RPMI 1640, and 100 μl of MTT (Sigma-Aldrich) solution (2.5 mg/ml in RPMI 1640 with 5% FCS) were added to each well. Plates were incubated for 50 min. Next, MTT solution was removed, and 50 μl of DMSO (Sigma-Aldrich) were added to each well to solubilize formazan crystals formed in viable cells. The absorbance was read at a wavelength of 590 nm on an ELISA plate reader.

Cytokine assay

Cells (1×10^5/ml) were cultured in RPMI 1640 containing 5% FCS, 2 mM L-glutamine, 0.1 mM NEAA, 100 IU/ml penicillin, and 100 μg/ml streptomycin with or without IL-17 or IL-17 plus 5 μg/ml anti-IL-17 mAb at indicated concentrations for 48 h. Cell-free supernatants were collected and stored at −70°C. Concentrations of cytokines were measured using commercially available ELISA kits (R&D Systems). PGE_2 concentration was measured as reported previously (36).

Migration assay

Migration of LMVECs was evaluated using a modified Boyden chamber assay, as described previously (37). Briefly, LMVECs were cultured in HuMedia-EB2 with 2% FCS for 8 h and plated at 12×10^4 cells/cm^2 onto the polycarbonate filter with 5-μm pores (Kurabo) coated with 10 μg/ml fibronectin (Sigma-Aldrich). CM from NSCLC unstimulated or stimulated with 50 ng/ml IL-17 (NSCLC/IL-17) was applied in the lower compartments of the chamber. LMVECs were cultured for 4 h at 37°C. The filters were fixed and stained with Diff-Quick (Harleco), and the number of migrating cells was quantified by counting cells in five randomly selected high-powered fields (HPFs) (×200) in each well. For the inhibitory assay, neutralizing mAb(s) (5 μg/ml each) was added in the upper and lower compartments of the chamber.

Construction of expression vector carrying human IL-17 cDNA

A *Bam*HI-*Xba*I fragment of the PCR product of IL-17 cDNA was ligated into the *Eco*RI site of pCRTM2.1. The insert DNA was excised from the plasmid with *Bam*HI/*Xba*I and inserted into the multiple cloning site of pRc/CMV. A549 and Sq-19 cells were transfected with the human IL-17 gene expression plasmid using LipofectAMINE (Invitrogen Life Technologies) and selected in RPMI 1640 with 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM NEAA, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 1000 μg/ml G418. As a control, a vector carrying only neomycin phosphotransferase gene (Neo) was used.

In vitro cell growth assay

To examine the in vitro growth, cells (5×10^4) were seeded in 10-cm culture plates on day 0, and the cell number was counted on days 2, 3, 4, 5, and 6.

Human NSCLC-SCID mouse chimeras

Eight- to 10-wk-old male SCID mice were inoculated s.c. with 7×10^6 Sq-19WT, Sq-19Neo, or Sq-19IL-17 cells or with 1×10^7 A549WT, A549Neo, or A549IL-17 cells into the right flank. The animals were maintained under sterile conditions in laminar flow rooms. In some experiments, mice were treated with either control Ab or rabbit polyclonal Ab specific for mouse CXCR-2 every 4 days. On day 25 for Sq-19 or on day 40 for A549, blood was collected from the mice, kept overnight at 4°C, and then centrifuged. The serum was stored at −70°C until use. Tumor volume (mm^3) was calculated using the formula $a \times b^2/2$ (a = largest diameter; b = smallest diameter) (38).

Immunohistochemical staining for Ki-67 in human NSCLC tissues from SCID mice

Analysis of the biological effect of IL-17 on the cell proliferation of NSCLC in vivo was performed with Ki-67 immunostaining, using MIB-1

Ab (Immunotech). Sections were deparaffinized and autoclaved in acetate buffer at 120°C for 5 min. After cooling, slides were incubated overnight at 4°C with MIB-1 or with normal mouse IgG (R&D Systems). After washing, sections were then incubated with biotinylated rabbit anti-mouse IgG and HRP-conjugated streptavidin (Nichirei, Tokyo, Japan). Sections were developed with 3,3′-diaminobenzidine (DAB) and counterstained with hematoxylin. The proliferating cells were estimated by the percentage of Ki-67-positive staining cells in 10 randomly chosen HPFs for each section (×400).

In situ detection of apoptosis

To detect apoptotic cells, the TUNEL method was performed using the WAKO in situ apoptosis detection kit (WAKO). Briefly, after deparaffinization, sections were incubated with a protein digestion enzyme for 5 min at 37°C. TdT with biotin-11-dUTP and dATP was then applied to the slides for 10 min at 37°C in a moist chamber. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 5 min at room temperature. Then, anti-biotin-11-dUTP labeling was conducted for 10 min at 37°C, followed by exposure to DAB. Sections were counterstained with methyl green. The apoptotic cells were estimated by the percentage of positive staining cells visualized in 10 randomly chosen HPFs for each section (×400).

Immunohistochemical staining for vascular endothelial cells in human NSCLC tissues and quantitation of vessel density

Immunostaining for CD31 was performed on frozen sections with rat anti-mouse CD31 mAb (BD Pharmingen). Briefly, frozen sections of NSCLC tissues from SCID mice were air-dried and incubated with 1% normal goat serum for 30 min at room temperature. Endogenous peroxidase activity was blocked using DakoCytomation peroxidase blocking reagent (DakoCytomation). Then, the primary Ab was applied overnight at 4°C. After washing, the sections were incubated with biotinylated rabbit anti-rat IgG Ab (DakoCytomation) for 30 min. After washing, peroxidase-conjugated streptavidin solution (DakoCytomation) was applied. Immunoreactivity was visualized by DAB and counterstained with hematoxylin.

Immunostaining for CD34 was performed on sections of NSCLC tissues from surgery, using the streptavidin-biotin amplification method (Histofine kit). Briefly, after deparaffinization, slides were treated with 3% hydrogen peroxidase in methanol for 10 min. Sections were then incubated with 1% normal goat serum for 30 min, followed by the application of mouse anti-human CD34 mAb (Nichirei) overnight at 4°C. Sections were treated with biotinylated anti-mouse IgG for 30 min, followed by peroxidase-conjugated streptavidin for 30 min. Immunoreactivity was visualized by DAB and counterstained with hematoxylin.

Sections were examined in a blinded fashion for the presence of CD31 or CD34 immunolocalization. According to the method described by Weidner et al. (39), specimens were scanned at low magnification (×40), and the 10 most vascularized tumor areas within a section were selected for evaluation of angiogenesis. Blood vessels stained with anti-mouse CD31 mAb or anti-human CD34 mAb were counted under light microscopy at ×200. Any distinct area of positive staining for CD31 or CD34 was counted as a single regardless of size. The results are shown as mean ± SD per 10 HPFs per tumor section.

Immunohistochemical staining for IL-17, IL-17R, and CD3 in human NSCLC tissues

Immunohistochemical staining for IL-17 and CD3 was performed on sections of NSCLC tissues from surgery, using the streptavidin-biotin amplification method (Histofine kit). Goat polyclonal Ab for human IL-17 and rabbit polyclonal Ab for human CD3 were used as a primary Ab. The dilution of primary Ab used in this study was 1/500 for IL-17 and 1/100 for CD3. Ag retrieval was performed by heating the slides in a microwave oven for 15 min for IL-17 or by treatment with protease K (Sigma-Aldrich) for 10 min for CD3. The Ag-Ab complex was visualized with DAB and counterstained with hematoxylin. As a positive control, we used the sections from blocks of Formalin-fixed, paraffin-embedded, IL-23-stimulated human CD4 T cells. As a negative control, an immunohistochemical preabsorption test was performed in these specimens.

For IL-17R staining, goat anti-human IL-17R polyclonal Ab (Santa Cruz Biotechnology) was used to characterize the expression of IL-17R on primary NSCLC tissues. As a control, sections were incubated with normal goat IgG (R&D Systems). The blocking of nonspecific proteins was conducted with PBS containing 2% BSA. The staining was conducted using Cy-3-conjugated rabbit anti-goat Ab as a secondary Ab (Sigma-Aldrich) and ProLong Gold antifade reagent with 4′,6′-diamidino-2-phenylindole (Molecular Probes) as a mounting medium. The pictures were captured by a camera attached to an Axioplan 2 universal imaging microscope (Intelligent Imaging Innovations) and were further analyzed with SlideBook 4.1 (Intelligent Imaging Innovations).

RT-PCR and quantitative RT-PCR

Total cellular RNA was extracted using Isogen (Nippon Gene) according to the manufacturer's instructions. Five micrograms of total RNA was used for the synthesis of cDNAs with SuperScript RNaseH-Reverse Transcriptase (Invitrogen Life Technologies). PCR was performed in a DNA Thermal Cycler (PerkinElmer/Cetus) using *Taq* polymerase (Boehringer Mannheim). The primer sequences of the oligonucleotides used for PCR and PCR product sizes were as follows: β-actin, sense, 5′-TCTGGTCAATG GAAGCCTGT-3′, and antisense, 5′-CTGTGGTGGTGAAGCTGTAC-3′, 436 bp; and human IL-17, sense, 5′-ACTCCTGGGAAGACCTCATTG-3′, and antisense, 5′-GGCCACATGGTGGACAATCG-3′, 461 bp. Real-time quantitative PCR (TaqMan PCR) using an ABI PRISM 7700 Sequence Detection System and TaqMan PCR Core Reagent kit (PerkinElmer) was performed according to the manufacturer's protocol. Oligonucleotides and a probe specific for human IL-17 were purchased from PerkinElmer Biosystems. Internal standard gene expression was examined by using TaqMan β-actin control reagents (PerkinElmer Biosystems). The amount of amplified IL-17 mRNA was correlated to that of β-actin mRNA.

Statistical analysis

Statistical analysis was performed using an unpaired two-tailed Student's *t* test with confirmation by parametric and *F* tests. Differences were considered to be statistically significant when the p value was <0.05.

Results

Expression of IL-17R on human NSCLC lines Sq-19, A549, and LK-87

The expression of IL-17R on three NSCLC cells was examined by flow cytometry using anti-human IL-17R mAb. These NSCLC cells expressed IL-17R on the surface at the protein level (Fig. 1). No staining was observed when an isotype matched control mAb was used (Fig. 1). These results are in accordance with the ubiquitous expression of IL-17R (2).

IL-17 has no direct effect on the in vitro growth of NSCLC cells

Because NSCLC cells expressed IL-17R, we investigated whether IL-17 can modulate the phenotype of these cells. We first examined the possibility that IL-17 might modulate the in vitro growth rate of NSCLC. To determine whether IL-17 affects the in vitro growth, cells were cultured in the presence or absence of human IL-17 for 5 or 7 days. A wide range of doses of IL-17 had no direct effect on the in vitro growth of Sq-19 and A549 cells as shown in Fig. 2, *A* and *B*. These results indicate that IL-17 has no biological action to promote or suppress the growth of NSCLC cells.

IL-17 selectively up-regulates the production of angiogenic CXC chemokines by three different NSCLC lines

We next examined the possibility that IL-17 might modulate the secretion of angiogenic and angiostatic factors by NSCLC, which promote or suppress tumor angiogenesis and growth. NSCLC cells have been reported to naturally produce diverse angiogenic factors. Interestingly, IL-17 selectively up-regulated the production of NSCLC-derived several angiogenic CXC chemokines, including CXCL1, CXCL5, CXCL6 and CXCL8 (Fig. 3, *A–C,* and Table I). Especially, IL-17 markedly augmented the production of CXCL1, CXCL5, and CXCL8 (Fig. 3, *A–C*). To control these results, we demonstrated that anti-IL-17 mAb inhibited the up-regulated production (Fig. 3, *A–C*), whereas an isotype-matched control mAb had no effect (data not shown). In contrast, IL-17 did not significantly enhance the production of acidic FGF (aFGF), bFGF, VEGF-A, PDGF-BB, HGF, TGF-α, angiopoietin-2, CXCL12, and PGE_2 (Fig. 3, *A–C*, and Table I). Moreover, we could not detect the up-regulated secretion of angiostatic CXC chemokines

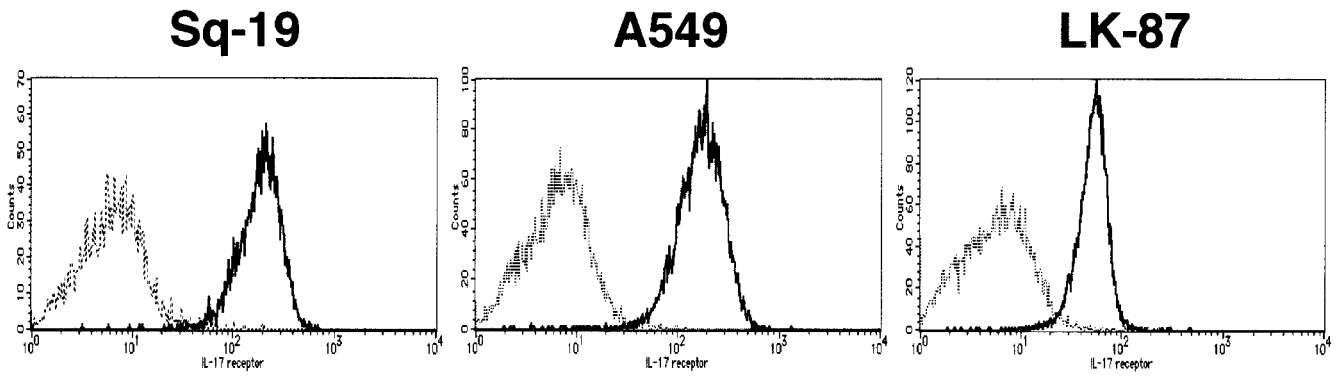

FIGURE 1. IL-17R expression on human NSCLC lines. Three different human NSCLC lines, Sq-19, A549, and LK-87, were incubated with mouse anti-human IL-17R mAb (solid line) or irrelevant mouse IgG1 (dotted line), followed by PE-conjugated goat anti-mouse IgG1 Ab. The expression of IL-17R on the surface of NSCLC cells was analyzed by flow cytometry.

CXCL9/monokine induced by IFN-γ, CXCL10, and CXCL11/IFN-inducible T cell α chemoattractant from NSCLC lines with IL-17 (Table I). These results indicate that IL-17 selectively up-regulates the production of an array of angiogenic CXC chemokines, but not angiostatic CXC chemokines, from NSCLC.

IL-17 significantly augments the net angiogenic activity of NSCLC

IL-17 selectively up-regulated the production of important angiogenic CXC chemokines of NSCLC. We therefore hypothesized that IL-17 might enhance the net angiogenic activity of NSCLC. To address this question, we performed endothelial cell chemotaxis assays on CM from NSCLC or NSCLC/IL-17. As shown in Fig. 4*A*, in comparison to CM from Sq-19, CM from Sq-19/IL-17 demonstrated a marked increase in angiogenic activity as assessed by endothelial cell chemotaxis. Similarly, CM from A549 demonstrated less angiogenic activity than CM from A549/IL-17 (Fig. 4*B*). These data clearly indicate that IL-17 significantly augments the net angiogenic activity of NSCLC measured as endothelial cell chemotactic activity.

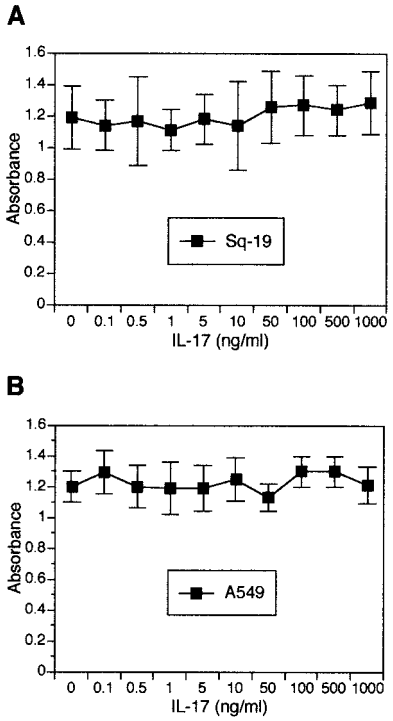

FIGURE 2. IL-17 has no direct effect on the in vitro growth of human NSCLC cells. Cells (1×10^3) were seeded into 96-well flat-bottom plates and cultured in RPMI 1640 with 3% FCS in the presence or absence of 0.1–1000 ng/ml human IL-17. On day 5 or 7, cells were washed with RPMI 1640, and 100 μl of MTT solution were added to each well. A wide range of doses of human IL-17 has no direct effect on the in vitro growth of Sq-19 and A549 NSCLC cells. Each value represents mean ± SD ($n = 7$). The result is representative of two independent experiments.

Increased endothelial cell chemotaxis in response to CM from NSCLC/IL-17 is suppressed in the presence of neutralizing mAb(s) to CXCL1, CXCL5, and CXCL8 or to CXCR-2

We hypothesized that the increased endothelial cell chemotaxis to CM from NSCLC/IL-17 was attributable to the observed increased secretion of angiogenic CXC chemokines by NSCLC with IL-17. To test this postulate, endothelial cell chemotaxis was performed with CM from NSCLC/IL-17 in the presence of neutralizing mAb(s) against CXCL1, CXCL5 and CXCL8, VEGF-A, HGF, IFN-γ, CXCR-1, or CXCR-2. We found that endothelial cell chemotaxis to CM from Sq-19/IL-17 was reduced significantly in the presence of neutralizing mAb(s) to CXCL1, CXCL5, and CXCL8 or to CXCR-2 as compared with control IgG (Fig. 5*A*). On the contrary, adding a neutralizing mAb against VEGF-A, HGF, IFN-γ, or CXCR-1 did not significantly inhibit the increased endothelial cell chemotaxis. Similarly, endothelial cell chemotaxis to CM from A549/IL-17 in the presence of neutralizing mAb(s) to CXCL1, CXCL5, and CXCL8 or to CXCR-2 was significantly less than that in the presence of control mouse IgG (Fig. 5*B*). These data demonstrate that IL-17 increases the net angiogenic activity of NSCLC through angiogenic CXC chemokine- and CXCR-2-dependent fashion.

Establishment and characterization of the IL-17-producing NSCLC lines

To evaluate the biological effect of IL-17 on NSCLC in detail, we generated the human IL-17 gene expression vector as described in the *Materials and Methods*. Two NSCLC lines, Sq-19 and A549, were transfected with human IL-17 gene using LipofectAMINE. No expression of either IL-17 mRNA or protein could be detected in Sq-19 and A549 cells before transfection (data not shown). After G418 selection, the expression of human IL-17 mRNA by stable transfectants was determined by RT-PCR (data not shown). Sq-19IL-17 or A549IL-17 secretes 67 or 35 ng/1 $\times$ 10^6 cells/48 h

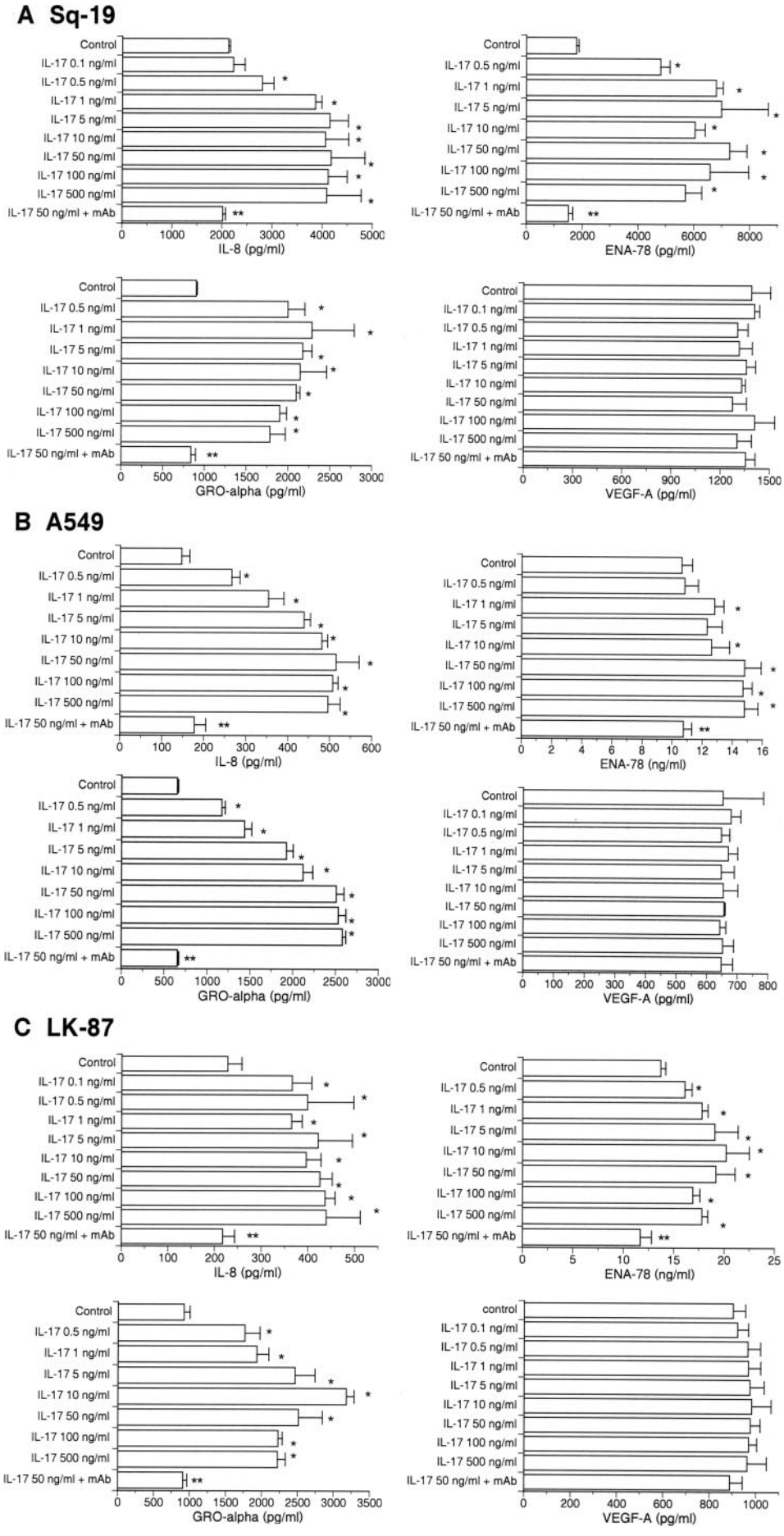

FIGURE 3. Effects of increasing concentrations of IL-17 on CXCL1, CXCL5, CXCL8, and VEGF-A production from human NSCLC lines. NSCLC lines, Sq-19 (*A*), A549 (*B*), and LK-87 (*C*), were stimulated with or without 0.1, 0.5 1, 5, 10, 50, 100, and 500 ng/ml IL-17 for 48 h. CXCL1, CXCL5, CXCL8, and VEGF-A concentrations in cell culture supernatants determined by ELISA are shown. Data are expressed as mean ± SD ($n = 3$). The result is representative of two independent experiments. (control vs 0.1–500 ng/ml IL-17, *, $p < 0.05$; 50 ng/ml IL-17 vs 50 ng/ml IL-17 + 5 μg/ml anti-human IL-17 mAb, **, $p < 0.003$).

Table I. *IL-17 selectively enhances the production of angiogenic CXC chemokines by human NSCLC cells*[a]

	Sq-19		A549		LK-87	
IL-17	−	+	−	+	−	+
aFGF	21.6 ± 0.5	22.4 ± 3.6	20.8 ± 0.9	17.7 ± 0.5	20 ± 2.0	21.9 ± 2.4
Basic FGF	<10	<10	47 ± 3	50 ± 3	17 ± 1	18 ± 1
Angiopoietin-2	132 ± 4	140 ± 21	157 ± 7	147 ± 11	133 ± 18	123 ± 11
TGF-α	8.9 ± 1.4	9.3 ± 2.1	13.9 ± 0.7	13.3 ± 0.7	23.7 ± 3.9	25.5 ± 4.3
HGF	150 ± 9	147 ± 14	178 ± 10	162 ± 17	135 ± 8	137 ± 6
CXCL1	0.9 ± 0.1	2.1 ± 0.1*	0.6 ± 0.1	2.5 ± 0.1*	0.9 ± 0.1	2.5 ± 0.3*
CXCL5	1.7 ± 0.1	7.3 ± 0.6*	10.6 ± 0.7	14.8 ± 1.1*	13.7 ± 0.5	19.2 ± 1.9*
CXCL6	3.6 ± 0.7	74.7 ± 10.5*	50.8 ± 7.0	91.7 ± 22.6*	23.0 ± 7.7	60.2 ± 6.0*
CXCL8	2.1 ± 0.1	4.2 ± 0.1*	0.15 ± 0.02	0.52 ± 0.05*	0.23 ± 0.03	0.43 ± 0.03*
CXCL10	10.7 ± 1.1	11.1 ± 0.7	37.7 ± 2.7	40.7 ± 3.7	17.1 ± 0.5	17.7 ± 1.5
CXCL11	<62.5	<62.5	<62.5	<62.5	<62.5	<62.5
CXCL12	79 ± 12	77 ± 37	93 ± 30	91 ± 5	64 ± 3	67 ± 5
PDGF-BB	367 ± 21	360 ± 8	337 ± 5	334 ± 7	330 ± 23	335 ± 7
PGE_2	4 ± 1	5 ± 1	65 ± 4	63 ± 7	108 ± 5	111 ± 7
VEGF-A	1.4 ± 0.1	1.3 ± 0.1	0.65 ± 0.13	0.66 ± 0.15	0.9 ± 0.1	1.0 ± 0.1

[a] Cells were cultured in RPMI 1640 containing 5% FCS, 2 mM L-glutamine, 0.1 mM NEAA, 100 IU/ml penicillin, and 100 μg/ml streptomycin with or without 50 ng/ml IL-17 for 48 h. Cell-free supernatants were collected and stored at −70°C. Concentrations of cytokines were measured using commercially available ELISA kits. PGE_2 concentration was measured as reported previously (36). (pg/ml for aFGF, bFGF, angiopoietin-2, TGF-α, HGF, CXCL6, CXCL10, CXCL11, CXCL12, PDGF-BB, and PGE2; ng/ml for CXCL1, CXCL5, CXCL8, and VEGF-A).

*, $p < 0.05$.

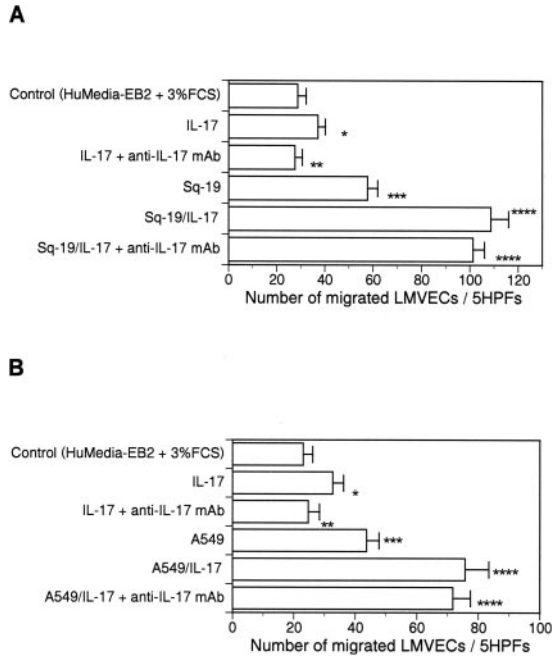

FIGURE 4. LMVEC chemotaxis to CM from Sq-19, Sq-19/IL-17, A549, or A549/IL-17. *A*, Endothelial cell chemotaxis to 50 ng/ml IL-17, CM from Sq-19, CM from Sq-19/IL-17, or CM from Sq-19/IL-17 + anti-IL-17 mAb. Bars represent the mean number of migrated LMVECs ± SD per five HPFs (×200) ($n = 5$). (Control vs IL-17, *, $p < 0.05$; IL-17 vs IL-17 + anti-IL-17 mAb, **, $p < 0.05$; control vs Sq-19, ***, $p < 0.01$; Sq-19 vs Sq-19/IL-17 or Sq-19/IL-17 + anti-IL-17 mAb; ****, $p < 0.003$.) *B*, Endothelial cell chemotaxis to 50 ng/ml IL-17, CM from A549, CM from A549/IL-17, or CM from A549/IL-17 + anti-IL-17 mAb. Bars represent the mean number of migrated LMVECs ± SD per five HPFs (×200) ($n = 5$). The result is representative of two independent experiments. (Control vs IL-17, *, $p < 0.05$; IL-17 vs IL-17 + anti-IL-17 mAb, **, $p < 0.05$; control vs A549, ***, $p < 0.02$; A549 vs A549/IL-17 or A549/IL-17 + anti-IL-17 mAb, ****, $p < 0.005$.)

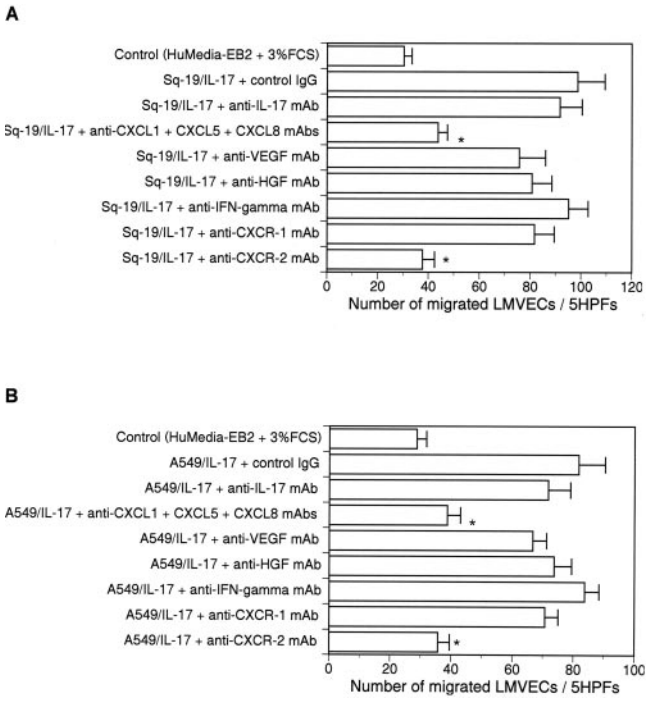

FIGURE 5. LMVEC chemotaxis to CM from NSCLC/IL-17 in the presence of mAb(s) to CXCL1, CXCL5 and CXCL8, VEGF-A, HGF, IFN-γ, CXCR-1 or CXCR-2. *A*, Endothelial cell chemotaxis to CM from Sq-19/IL-17 in the presence of neutralizing mAb(s) to CXCL1, CXCL5 and CXCL8, VEGF-A, HGF, IFN-γ, or CXCR-2. Bars represent the mean number of migrated LMVECs ± SD per five HPFs (×200) ($n = 5$). The result is representative of two independent experiments. (Control IgG vs anti-CXCL1, CXCL5 and CXCL8 mAbs, or anti-CXCR-2, *, $p < 0.003$). *B*, Endothelial cell chemotaxis to CM from A549/IL-17 in the presence of neutralizing mAb(s) to CXCL1, CXCL5 and CXCL8, VEGF-A, HGF, IFN-γ, or CXCR-2. Bars represent the mean number of migrated LMVECs ± SD per five HPFs (×200) ($n = 5$). The result is representative of two independent experiments. (Control IgG vs anti-CXCL1, CXCL5, and CXCL8 mAbs or anti-CXCR-2, *, $p < 0.007$.)

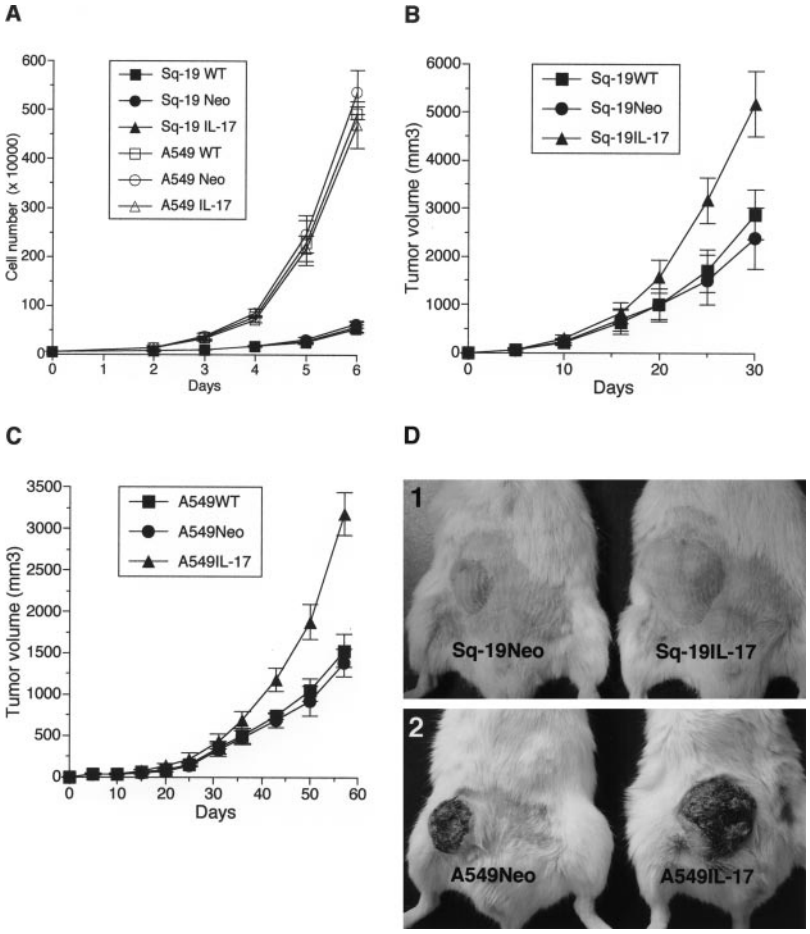

FIGURE 6. Expression of IL-17 markedly promotes the in vivo NSCLC growth in SCID mice. *A*, Transduction with the IL-17 gene has no direct effect on the in vitro growth of NSCLC cells. Each value represents mean ± SD ($n = 3$). The result is representative of two independent experiments. *B*, The time course of the in vivo growth for Sq-19WT, Sq-19Neo, and Sq-19IL-17 in SCID mice. Data are mean tumor volume ± SD for seven mice per group. The result is representative of two independent experiments. *C*, The time course of the in vivo growth for A549WT, A549Neo, and A549IL-17 in SCID mice. Data are mean tumor volume ± SD for seven mice per group. The result is representative of two independent experiments. *D*, These are representative photographs of the gross NSCLC tumors in SCID mice.

IL-17, respectively, determined by a commercially available ELISA kit (R&D Systems).

IL-17 significantly promotes the in vivo growth of NSCLC in SCID mice

Significant changes were not observed in the in vitro growth of IL-17 transfectants when compared with that of parental cells or Neo transfectants (Fig. 6*A*). When Sq-19WT, Sq-19Neo, or Sq-19IL-17 cells were implanted s.c. in SCID mice, they all formed solid tumors. However, Sq-19IL-17 developed tumors with a strikingly increased growth rate compared with controls (Sq-19WT vs Sq-19 IL-17: $p < 0.0003$; Sq-19Neo vs Sq-19IL-17: $p < 0.0002$, on day 30) (Fig. 6, *B* and *D*). A similar increase in in vivo tumor growth was observed when A549IL-17 was implanted in SCID mice as compared with controls (A549WT vs A549 IL-17: $p < 0.0007$; A549Neo vs A549 IL-17: $p < 0.0006$, on day 45) (Fig. 6, *C* and *D*). In in vitro cultures, A549 proliferated more rapidly than Sq-19, whereas Sq-19 grew much faster in vivo than A549 when transplanted in SCID mice. The representative photographs of the gross NSCLC tumors in SCID mice were shown in Fig. 6*D*.

IL-17 significantly increases the intratumoral microvessel density

Although IL-17 enhanced the in vivo growth of NSCLC, the result raised the question as to how IL-17 promotes the in vivo growth of these tumors, inasmuch as IL-17 has no direct effect on the proliferation of NSCLC cells in vitro. To address this question and further investigate the mechanism by which the in vivo growth of NSCLC was enhanced with IL-17, NSCLC tumors were excised, and the proliferation index of tumor cells in situ was quantified by immunohistochemical staining for Ki-67 (Fig. 7, *A–D*). Although the volume of NSCLC tumors transduced with IL-17 increased to ~170% of the controls, the proliferation index did not significantly change (Fig. 7*M*). Thus, we speculated that IL-17 increases the in vivo growth by promoting tumor angiogenesis rather than stimulating the proliferation of NSCLC cells. To examine this possibility, we evaluated the vascular density by immunohistochemical examination of NSCLC tissues. Immunostaining for CD31 showed that tumor tissues of IL-17 transfectants were more markedly vascularized when compared with those of controls (Fig. 7, *I–L*). To compare the vascular density, the mean number of blood vessels of CD31-stained sections obtained from five independent tumors was determined. The mean number of microvessels of Sq-19IL-17 tumors was significantly higher than that of Sq-19WT or Sq-19Neo on day 25 ($p < 0.007$) (Fig. 7*M*). Similar results were obtained in A549 tumors on day 40 ($p < 0.001$) (Fig. 7*M*). These results indicate that the enhanced in vivo growth of IL-17 transfectants closely correlated with increased vascularity. Furthermore, the TUNEL assay showed that IL-17 led to a 0.34-fold decrease for Sq-19 and 0.47-fold decrease for A549 in the number of apoptotic cells (Fig. 7*M*). These findings are consistent with previous studies, which showed that angiogenesis stimulators or inhibitors can modulate the in vivo tumor growth by increasing or decreasing apoptosis of tumor cells (40).

The elevated levels of circulating serum angiogenic chemokines during tumorigenesis of NSCLC cells transfected with IL-17 in SCID mice correlate with increased growth

Because IL-17 selectively augments the secretion of angiogenic CXC chemokines by NSCLC and promotes tumor angiogenesis,

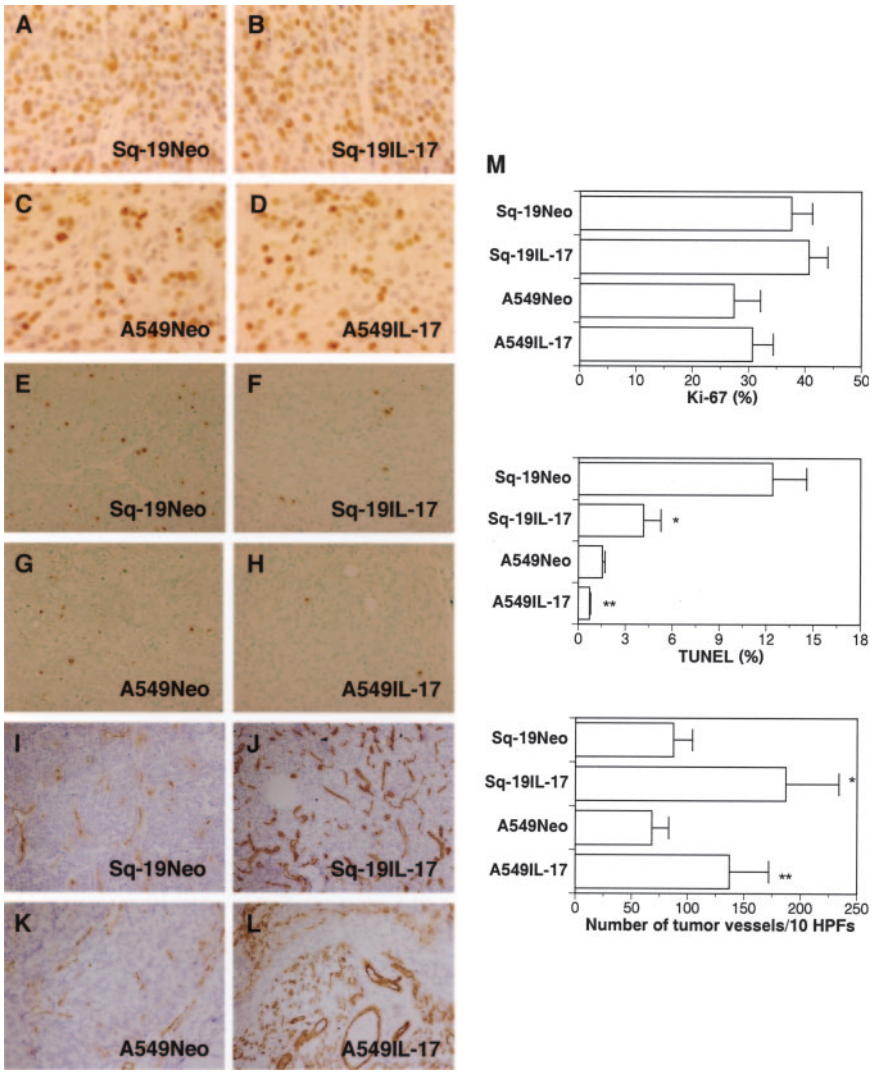

FIGURE 7. Immunohistochemical examination of NSCLC tissues. Tumor tissues were excised from SCID mice, fixed in 4% buffered paraformaldehyde, embedded in paraffin or immediately soaked in OCT compound, and frozen in liquid nitrogen. Representative photomicrographs show a typical immunochemical appearance in tumor tissues from Neo transfectants (*A*, *C*, *E*, *G*, *I*, and *K*) and IL-17 transfectants (*B*, *D*, *F*, *H*, *J*, and *I*). Proliferation, apoptosis and angiogenesis were detected using anti-Ki-67 Ab (*A–D*), the TUNEL method (*E–H*), and anti-CD31 mAb (*I–L*), respectively. *M*, Graphs show the change in Ki-67-positive cells, TUNEL-positive cells, and blood vessel number in the tumor tissues. Data represent the mean ± SD ($n = 5$).

we postulated that the increased growth of NSCLC with IL-17 was mediated by an enhanced production of angiogenic chemokines. Thus, we initially examined the serum concentrations of angiogenic CXC chemokines in SCID mice bearing NSCLC. As shown in Table II, the serum concentrations of CXCL1, CXCL5, and CXCL8 in mice bearing IL-17 transfectants increased in direct correlation with enlarged tumor size.

Administration of anti-mouse CXCR-2-neutralizing Ab into tumor-bearing SCID mice markedly abrogates the IL-17-induced increased growth and vascularity

To delineate the role for up-regulated production of angiogenic chemokines during tumorigenesis of IL-17 transfectants, animals were subjected to a strategy to block the biological action of angiogenic CXC chemokines. Because the CXC chemokine receptor 2, CXCR-2, has been reported to be the receptor accounting for CXC chemokine-induced angiogenic activity on human and mouse endothelium (41), SCID mice were treated with either control Ab or neutralizing Ab against mouse CXCR-2 at the time of inoculation and every 4 days for a period of 4 or 5 wk. As illustrated in Fig. 8*A*, Sq-19IL-17 tumor-bearing SCID mice treated with anti-CXCR-2 Ab demonstrated a marked reduction in tumor growth as compared with animals that were treated with control irrelevant Ab (control Ab vs anti-CXCR-2 Ab; $p < 0.0001$, on day 30). In addition, Sq-19Neo tumors in SCID mice treated with anti-CXCR-2 Ab grew more slowly than those in animals that were treated with control Ab (control Ab vs anti-CXCR-2 Ab; $p < 0.007$, on day 30). Similarly, administration of anti-CXCR-2 Ab into SCID mice with A549IL-17 tumors resulted in a significant reduction of tumor growth (control Ab vs anti-CXCR-2 Ab; $p < 0.0003$, on day 55) (Fig. 8*B*). Moreover, treatment with anti-CXCR-2 Ab significantly suppressed the growth of A549Neo in SCID mice (control Ab vs anti-CXCR-2 Ab; $p < 0.01$, on day 55). We also investigated the microvessel density and found that the treatment with anti-CXCR-2 Ab significantly reduced the tumor vascularity of Neo transfectants as well as IL-17 transfectants (Table III). These results indicate that the increased growth of NSCLC elicited by IL-17 is mediated by CXCR-2 signaling and that NSCLC growth

Table II. *Markedly elevated serum concentrations of angiogenic CXC chemokines in SCID mice bearing tumors transfected with IL-17*[a]

Tumors	CXCL1	CXCL5	CXCL8
Sq-19Neo	475.5 ± 117.7	717.5 ± 171.7	117.5 ± 37.1
Sq-19IL-17	1577.2 ± 317.1*	1877.8 ± 517.5*	233.8 ± 51.7*
A549Neo	175.7 ± 48.7	1657.7 ± 177.5	51.7 ± 13.7
A549IL-17	575.7 ± 138.7*	2146.7 ± 217.7*	205.2 ± 34.3*

[a] On day 25 for Sq-19 or on day 40 for A549, peripheral blood was collected from SCID mice, kept over night at 4°C, and centrifuged. The serum was stored at −70°C until use. The circulating levels of angiogenic CXC chemokines were measured using commercially available ELISA kits. Data are the mean ± SD (pg/ml) ($n = 3$). The result is representative of two independent experiments. *, $p < 0.05$.

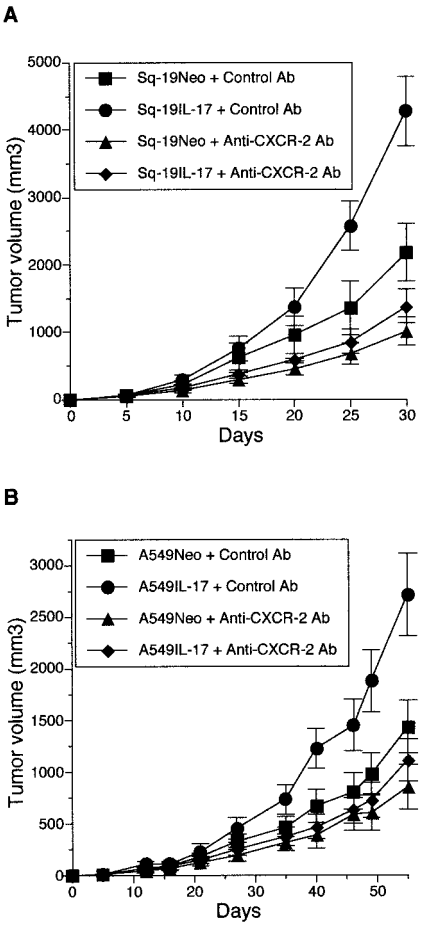

FIGURE 8. Administration of anti-mouse CXCR-2-neutralizing Ab into SCID mice significantly attenuates the in vivo growth of NSCLC. SCID mice were inoculated s.c. with NSCLC cells and treated with either control Ab or anti-mouse CXCR-2 Ab at 4-day intervals. *A*, The time course of in vivo growth for Sq-19Neo and Sq-19IL-17 in SCID mice treated with either control Ab or anti-mouse CXCR-2 Ab. Data are mean tumor volume ± SD for five mice per group. The result is representative of two independent experiments. *B*, The time course of in vivo growth for A549Neo and A549IL-17 in SCID mice treated with either control Abs or anti-mouse CXCR-2 Ab. Data are mean tumor volume ± SD for five mice per group. The result is representative of two independent experiments.

is partly dependent on angiogenic CXC chemokines, which commonly bind to CXCR-2. These findings also suggest that the blocking of CXCR-2 signaling might be a promising strategy to treat patients with NSCLC.

IL-17 production and vascularity in primary NSCLC tissues

To further explore the possible role for IL-17 in promoting angiogenic activity of NSCLC, we investigated the IL-17 mRNA expression in freshly isolated surgical specimens of NSCLC and found that its expression was detected in 57% of the cases (44 of 77 samples) (Fig. 9*A* and data not shown). The levels of IL-17 mRNA expression assessed by quantitative RT-PCR were classified as follows: high > 0.15, low $\leqq 0.15$, and undetectable $\leqq 0.001$. High levels of IL-17 expression were found in 17 cases, low in 27, and undetectable in 33. To investigate what types of cells produce IL-17 in primary NSCLC tissues, we performed an immunohistochemical analysis using anti-human IL-17 Ab. The strong immunoreactivity for IL-17 was found when high levels of IL-17 expression were detected, whereas the immunoreactivity was not observed when IL-17 expression was not detected (Fig. 9, *D* and *E*). Furthermore, in NSCLC tissues, the immunoreactivity for IL-17 was found only in the accumulating and infiltrating inflammatory cells but not in tumor cells (Fig. 9*E*). Fossiez et al. (7) reported that IL-17 transcripts are detected only in T cells upon activation. To examine whether the infiltrating T cells are the origin of IL-17 production, we stained the serial sections of NSCLC tissues using Ab against IL-17 or CD3. Surprisingly, the cells stained positively with anti-IL-17 Ab were not necessarily stained with anti-CD3 Ab (Fig. 9, *F* and *G*). These cells, which were stained positively with anti-IL-17 Ab and negatively with anti-CD3 Ab, had polymorphonuclear morphology (Fig. 9*H*). These findings indicated that, in NSCLC tissues, the IL-17-producing cells are T cells and polymorphonuclear neutrophils. In addition, we found that the presence of T cells in NSCLC tissues had no direct correlation with IL-17 expression. In some NSCLC cases, infiltrating T cells were stained positively with anti-IL-17 Ab, whereas, in other cases, T cells were not stained at all (data not shown). Moreover, there was no significant relationship between IL-17 expression and any clinicopathologic parameter such as stage or histological type (data not shown).

We also examined the expression of IL-17R in NSCLC tissues from surgery by immunohistochemical staining. The strong immunoreactivity for IL-17R on NSCLC cells was detected (Fig. 9, *I* and *J*). We further examined the vascular density of primary NSCLC tissues by immunostaining using anti-human CD34 mAb. A statistically significant association was found between high levels of IL-17 expression and tumor vascularity (Fig. 9, *K* and *L*). The vascular density of NSCLC tissues with high levels of IL-17 expression was significantly higher than that of NSCLC tissues with undetectable levels of IL-17 (Table IV).

Table III. *Treatment with anti-mouse CXCR-2-neutralizing Ab significantly reduced the intratumoral microvessel density*[a]

Treatment	No. of Tumor Vessels/10 HPFs
Sq-19Neo + control Ab	81 ± 11
Sq-19Neo + anti-CXCR-2 Ab	57 ± 7*
Sq-19IL-17 + control Ab	171 ± 31
Sq-19IL-17 + anti-CXCR-2 Ab	64 ± 8**
A549Neo + control Ab	75 ± 10
A549Neo + anti-CXCR-2 Ab	47 ± 5***
A549IL-17 + control Ab	130 ± 17
A549IL-17 + anti-CXCR-2 Ab	56 ± 6****

[a] Tumor tissues were harvested from SCID mice on day 25 for Sq-19 or on day 40 for A549, immediately soaked in OCT compound, and frozen in liquid nitrogen. Sections were stained for CD31. Specimens ($n = 4$) were evaluated by quantifying the number of stained blood vessels in 10 selected most vascularized HPFs per tumor section. The mean number of microvessels/10 HPFs/tumor section (×200) from mice treated with anti-mouse CXCR-2-neutralizing Ab was much less than that from mice treated with control Ab. Data represent the mean number of vessels ± SD. (Sq-19Neo + control Ab versus Sq-19Neo + anti-CXCR-2 Ab, *, $p < 0.01$; Sq-19IL-17 + control Ab vs Sq-19IL-17 + anti-CXCR-2 Ab, **, $p < 0.002$; A549Neo + control Ab vs A549Neo + anti-CXCR-2 Ab, ***, $p < 0.01$; A549IL-17 + control Ab vs A549IL-17 + anti-CXCR-2 Ab, ****, $p < 0.005$).

Discussion

In the present study, we demonstrate that IL-17, a T cell cytokine, markedly increases the net angiogenic activity and promotes the in vivo growth of NSCLC transplanted in SCID mice through a CXCR-2-dependent mechanism. We found that IL-17 stimulates NSCLC to selectively up-regulate the production of an array of angiogenic, but not angiostatic, CXC chemokines and strikingly enhances the NSCLC-derived net angiogenic activity. Although two NSCLC lines transfected with the IL-17 gene grew more rapidly when compared with controls in SCID mice, administration of anti-mouse CXCR-2-neutralizing Ab into mice largely abolished

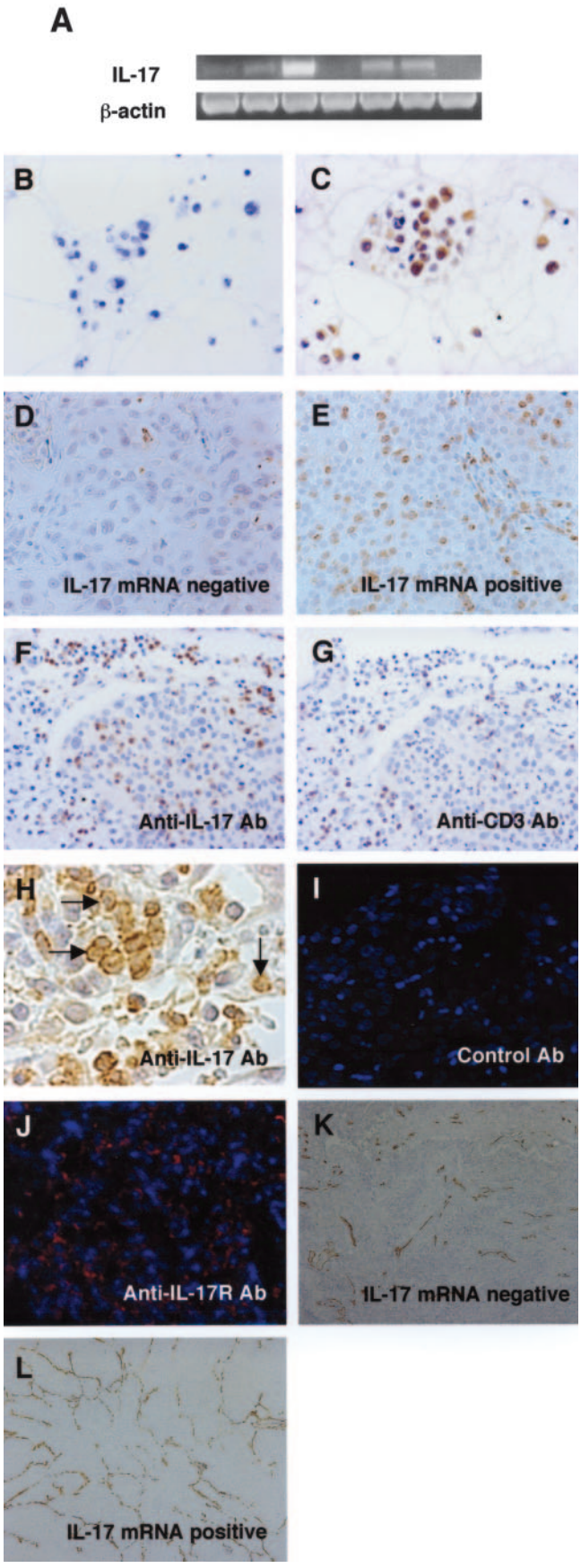

FIGURE 9. IL-17 and IL-17R expression and vascularity in human NSCLC tissues. *A*, Total cellular RNA was extracted from human NSCLC tissues. Five micrograms of total RNA were applied for the synthesis of cDNAs. IL-17 mRNA expression was detected in five of seven NSCLC cases by RT-PCR. *Lanes 1*, *3*, *4*, *5*, and *7* are IL-17 mRNA positive. *B* and *C*, As a positive control, we stained the sections from blocks of formalin-fixed and paraffin-embedded, IL-23-stimulated CD4 T cells with anti-human IL-17 Ab. As a negative control, immunohistochemical preabsorption test was performed in these sections. *D*, Anti-IL-17 reactivity in NSCLC tissues with undetectable levels of IL-17 mRNA expression. The immunoreactivity for IL-17 is not observed. *E*, Anti-IL-17 reactivity in NSCLC tissues with high levels of IL-17 mRNA expression. The strong immunoreactivity for IL-17 is observed in infiltrating inflammatory cells but not in NSCLC cells. *F* and *G*, The serial sections of NSCLC tissues were stained with Ab against IL-17 (*F*) or CD3 (*G*). Not all cells, which are stained positively with anti-IL-17 Ab, are stained positively with anti-CD3 Ab. *H*, Some of the cells stained positively with anti-IL-17 Ab have polymorphonuclear morphology. *I* and *J*, IL-17R expression in NSCLC tissues from surgery was examined by immunohistochemical staining using goat anti-human IL-17R polyclonal Ab. The strong immunoreactivity for IL-17R is detected on the surface of NSCLC cells. *K* and *L*, NSCLC tissues from surgery were fixed in 4% buffered paraformaldehyde and embedded in paraffin. NSCLC sections were stained with anti-CD34 mAb and counterstained with hematoxylin. NSCLC tissues with high levels of IL-17 mRNA expression are more markedly vascularized than those with undetectable levels of IL-17 expression.

Table IV. *Relation between vascular density and IL-17 mRNA expression in NSCLC tissues*[a]

IL-17 mRNA Expression	Vascular Density
Undetectable ($n = 33$)	175.4 ± 15.7
High ($n = 17$)	217.7 ± 27.4*

[a] NSCLC sections were stained for CD34. Specimens were evaluated by quantifying the number of stained blood vessels/10 HPFs/section (×200). Data represent the mean number of vessels ± SD. (Undetectable levels of IL-17 mRNA expression versus high levels of IL-17 mRNA expression; *, $p < 0.03$).

this enhanced NSCLC growth. A direct effect of IL-17 on the in vivo growth of NSCLC cells seems unlikely because a wide range of doses of IL-17 did not affect the in vitro growth rate and wild-type, Neo-, or IL-17-transfected tumors exhibited the same in vitro proliferation rate. Immunostaining for CD31 revealed that the vascular elements within tumor tissues of IL-17 transfectants significantly increased when compared with those of controls. Because angiogenesis is an essential process in the development and progression of malignant solid tumors, our findings strongly suggest that IL-17 might accelerate the in vivo NSCLC growth via enhancing the net angiogenic activity.

The CXC chemokines can be divided into two groups on the basis of the presence or absence of the glutamic acid-leucine-arginine (ELR) motif. CXC chemokines with the ELR motif are potent angiogenic factors that directly promote endothelial cell proliferation, chemotaxis, and tubular morphogenesis (42, 43). ELR^+ CXC chemokines are also major angiogenic factors in NSCLC and murine models of human NSCLC (33–34, 44). In contrast, CXC chemokines without the ELR motif such as CXCL9 and CXCL10 are potent angiostatic factors (45). Especially, CXCL10 is an important endogenous angiostatic factor in NSCLC, which negatively regulates the NSCLC-derived net angiogenic activity (35). The relative expression of angiogenic, as compared with angiostatic, members of the CXC chemokine family is an important determinant of the net angiogenic activity in NSCLC (24, 46). The IFNs such as IFN-α, IFN-β, and IFN-γ are potent agonists for the expression of CXCL10 from a variety of cells, including keratinocytes, fibroblasts, endothelial cells, mononuclear cells, and tumor cells (47–49). The IFNs are also potent inhibitors of the production of CXCL8 by monocytes, fibroblasts, and endothelial cells (50–

53). Conversely, IL-17 is a potent inducer of angiogenic chemokines from a number of cells, including keratinocytes, fibroblasts, epithelial cells, and tumor cells (2–8). IL-17 also inhibits TNF-α-induced CXCL10 secretion by fibroblasts (54). In this study, we indicated that IL-17 has the capability to selectively enhance the production of angiogenic CXC chemokines from NSCLC. Taken together, the IFNs may shift the local biologic balance between angiogenic and angiostatic CXC chemokines toward a predominance of angiostatic chemokines to reduce the net angiogenic activity, whereas IL-17 may promote the angiogenic activity of NSCLC by causing a biological imbalance to increase the production of angiogenic CXC chemokines and to suppress the secretion of angiostatic CXC chemokines.

To confirm the action of IL-17 in enhancing the net angiogenic activity of NSCLC via selectively up-regulated production of angiogenic CXC chemokines, we performed an endothelial cell chemotaxis assay on CM from NSCLC/IL-17. IL-17 markedly promoted NSCLC-induced endothelial chemotactic activity. To assess whether this biological property of IL-17 could be caused by angiogenic chemokines, we tested the inhibitory effects of neutralizing mAb(s) against angiogenic molecule(s) or their receptor and found that the inhibition of CXCL1, CXCL5, and CXCL8 or CXCR-2 abolished the enhanced endothelial cell chemotaxis to CM from NSCLC/IL-17. On the contrary, neutralization of VEGF-A, HGF, IFN-γ, or CXCR-1 did not significantly affect the increased endothelial chemotaxis in the presence of IL-17. Thus, it is confirmed that the enhanced angiogenic activity of NSCLC stimulated with IL-17 is mediated by an array of angiogenic CXC chemokines.

Because neutralization of angiogenic CXC chemokines or their receptor CXCR-2 in vivo could constitute the direct evidence of the role for these factors as mediators of IL-17, we administered the neutralizing anti-mouse CXCR-2 Ab into mice. Treatment with anti-CXCR-2 Ab led to a marked reduction in in vivo growth of IL-17 transfectants, confirming the findings in in vitro endothelial cell chemotaxis assay. Interestingly, treatment with anti-CXCR-2 Ab significantly suppressed the neovascularization and in vivo growth of Neo transfectants as well as IL-17 transfectants. These findings are consistent with the previous report demonstrating that the in vivo growth of Lewis lung cancer primary tumors significantly reduced in CXCR-$2^{-/-}$ mice as compared with that in control mice (55). For antiangiogenic therapy to be successful, it must inhibit diverse angiogenic stimuli produced by the tumor and its microenvironment. Thus, the attractive feature of targeting the common receptor CXCR-2 for the angiogenic CXC chemokines is the fact that it results in the inhibition of binding of several ELR^+ chemokine ligands at once. From this point of view, targeting CXCR-2 signaling may be more effective than other monotherapies, which target a single mediator such as CXCL8, to treat patients with NSCLC. Moreover, combination antiangiogenesis protocols, which target both CXCR-2 and VEGF-A signalings, could greatly improve the therapeutic efficacy of NSCLC. Taken collectively, our results indicate that CXCR-2 signaling mainly mediates the angiogenic activity of NSCLC and highlights the importance of developing the novel strategies to target CXCR-2 signaling.

In the current study, we showed that IL-17 expression is frequently detected in NSCLC tissues. Moreover, our immunohistochemical analysis provided an additional evidence that, in NSCLC tissues, the infiltrating inflammatory cells, but not tumor cells, are producing IL-17. There have been reports demonstrating that T cells infiltrating into cervical and prostate carcinoma tissues are expressing IL-17 (56, 57). Interestingly, in NSCLC tissues, polymorphonuclear neutrophils in addition to T cells are producing IL-17. In addition, IL-17R is expressed on the surface of primary NSCLC cells. Thus, it is likely that IL-17, produced by both T cells and polymorphonuclear neutrophils in situ, may have some biological action on NSCLC cells in a paracrine fashion. Immunostaining for CD34 revealed that NSCLC tissues with high levels of IL-17 mRNA expression had significantly higher microvessel density than those in which IL-17 mRNA expression was not detected. These findings raised the possibility that the infiltrating inflammatory cells such as T cells and polymorphonuclear neutrophils may occasionally stimulate the production of angiogenic CXC chemokines by NSCLC via secretion of IL-17.

We recently reported that IL-17 is an angiogenic factor, which stimulates the migration and cord formation of vascular endothelial cells in vitro and elicits neovessel formation in vivo (37, 58). Thus, it is a little strange that the enhanced NSCLC growth mediated by IL-17 was largely impaired in mice treated with anti-CXCR-2 Ab. Although the elucidation of a precise mechanism by which IL-17 mediates angiogenesis in vivo is far from complete, neovessel formation elicited by IL-17 may be mostly mediated by the angiogenic CXC chemokine family, which shares a common chemokine receptor CXCR-2.

Although our study indicates that CD4 T cell cytokine may promote the in vivo NSCLC growth through enhancing CXCR-2-dependent angiogenesis, CD4 T cells have been thought, in general, to regulate angiogenesis negatively via production of IFN-γ, which in turn induces the production of angiostatic CXC chemokines (59, 60). CD4 T cells stimulated by IL-12 secrete IFN-γ and inhibit angiogenesis (61). In contrast, physiological regulation of IL-17 production by CD4 T cells has not been fully elucidated (62, 63). Taken collectively, our results suggest that CD4 T cells may have the regulatory ability to promote or suppress angiogenesis depending on the stimuli.

In conclusion, our findings illustrate the biological action of IL-17 on human NSCLC. IL-17 markedly enhances the net angiogenic activity and promotes the in vivo growth of NSCLC via selectively up-regulated production of an array of angiogenic CXC chemokines, which lead to an imbalance between angiogenesis promoters and inhibitors present within the vascular microenvironment. Our results also demonstrate that targeting CXCR-2 signaling may represent a potential therapeutic strategy against NSCLC. Further analyses are needed to elucidate the precise role for IL-17 in tumor angiogenesis and growth of NSCLC.

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Disclosures

The authors have no financial conflict of interest.

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