High Expression of Ligands for Chemokine Receptor CXCR2 in Alveolar Epithelial Neoplasia Induced by Oncogenic Kras

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Abstract

CXCL8, a ligand for the chemokine receptor CXCR2, was recently reported to be a transcriptional target of Ras signaling, but its role in Ras-induced tumorigenesis has not been fully defined. Here, we investigated the role of KC and MIP-2, the murine homologues of CXCL8, in KrasLA1 mice, which develop lung adenocarcinoma owing to somatic activation of the Kras oncogene. We first investigated biological evidence of CXCR2 ligands in KrasLA1 mice. Malignant progression of normal alveolar epithelial cells to adenocarcinoma in KrasLA1 mice was associated with enhanced intraluminal vascularity and neutrophilic inflammation, which are hallmarks of chemoattraction by CXCR2 ligands. In vitro migration assays, supernatants of bronchoalveolar lavage samples from KrasLA1 mice chemotacted murine endothelial cells, alveolar inflammatory cells, and the LKR-13 lung adenocarcinoma cell line derived from KrasLA1 mice, an effect that was abrogated by pretreatment of the cells with a CXCR2-neutralizing antibody. CXCR2 and its ligands were highly expressed in LKR-13 cells and premalignant alveolar lesions in KrasLA1 mice. Treatment of KrasLA1 mice with a CXCR2-neutralizing antibody inhibited the progression of premalignant lesions and induced apoptosis of vascular endothelial cells within alveolar lesions. Whereas the proliferation of LKR-13 cells in vitro was resistant to treatment with the antibody, LKR-13 cells established as syngeneic tumors were sensitive, supporting a role for the tumor microenvironment in the activity of CXCR2. Thus, high expression of CXCR2 ligands may contribute to the expansion of early alveolar neoplastic lesions induced by oncogenic Kras. (Cancer Res 2006; 66(8): 4198-207)

Introduction

Non–small cell lung cancer (NSCLC) is highly invasive and frequently metastatic at the time of diagnosis. Because metastatic NSCLC is refractory to all known interventions, a logical approach to reducing mortality from NSCLC is to intervene before the development of invasive disease. Success in this effort will require an understanding of the genetic and biochemical changes in NSCLC that confer invasive properties.

The biological features of NSCLC associated with advanced stages of disease include neutrophil infiltration and enhanced tumor vasculature (1–4). Neutrophils and vascular endothelial cells are recruited to tumors by the class of CXC chemokines with an NH2-terminal Glu-Leu-Arg (ELR) motif (5–10). These chemokines are also autocrine growth factors for certain types of cancer cells (11–13). There are three known receptors for these chemokines: CXCR1 and CXCR2, which are G-protein-coupled receptors, and Duffy antigen receptor. Endothelial cells, as well as other cell types, express CXCR2, thereby promoting angiogenesis in tumors that express ELR-positive CXC chemokines (14–17).

CXCL8, also known as interleukin-8 (IL-8), is a CXCR2 ligand that is present in freshly isolated specimens of human NSCLC (18). Two murine functional homologues of CXCL8 (KC and MIP-2) have been implicated as the dominant mediators of aberrant angiogenesis in a syngeneic murine Lewis lung cancer model and in a human NSCLC/severe combined immunodeficient mouse chimera (5, 15). A recent study showed that CXCL8 is a transcriptional target of Ras signaling and is required for the initiation of tumor-associated inflammation and neovascularization in xenograft models (19). Mutations in the proto-oncogene Kras occur in 30% to 50% of lung adenocarcinomas, the most common subtype of NSCLC, and expression of mutant KRAS in the alveolar epithelium leads to the development of lung adenocarcinoma in mice (20–23). In addition to its role in the transformation of alveolar epithelial cells, the presence of KRAS mutations is a predictor of shorter survival in NSCLC patients (24).

Based on recent evidence that CXCL8 plays a crucial role in the establishment of Ras-induced tumors (19), we sought to determine whether CXCR2 ligands contribute to malignant progression in a relevant model of KRAS-induced lung tumorigenesis. We investigated KrasLA1 mice, which develop lung adenocarcinoma through somatic activation of a KRAS allele carrying an activating mutation in codon 12 (G12D; ref. 23). Alveolar epithelial cells in this mouse model recapitulate the series of morphologic stages through which human atypical alveolar hyperplasia (AAH) evolves into adenocarcinoma. We previously reported prominent infiltrates of macrophages in premalignant alveolar lesions of KrasLA1 mice (25), supporting the presence of chemokines at early stages of lung tumorigenesis. We found neutrophils, vascular endothelial cells, and high expression of KC, MIP-2, and CXCR2 in premalignant...
alveolar lesions of Kras<sup>A1</sup> mice. CXCR2 inhibition blocked the expansion of early alveolar neoplastic lesions, and the antitumor effect of CXCR2 inhibition required the presence of the tumor microenvironment. Thus, high expression of CXCR2 ligands may be an important early event in alveolar neoplasia induced by oncogenic KRAS.

Materials and Methods

Animal experiments. Animal experiments were done in compliance with the guidelines of The University of Texas M.D. Anderson Cancer Center. Kras<sup>A1</sup> mice were provided by Dr. Tyler Jacks (Massachusetts Institute of Technology, Cambridge, MA). For the CXCR2 neutralization experiments, 16-week-old Kras<sup>A1</sup> mice were given 500 μL of CXCR2 immune serum (CIS; n = 13) or normal goat preimmune serum (NGS; n = 14) by i.p. injection thrice a week for 3 weeks as previously described (15). Mice were subjected to respiratory-gated micro-computed tomography (micro-CT) at the beginning and end of the treatment (26), and they were killed by cervical dislocation within 6 hours of the final scan. During the autopsy, visible lesions were counted on the surfaces of both lungs by investigators (M.W. and A.H.) blinded to the treatment group. The lungs were then perfused with PBS and washed from the body. One lung was kept at −80°C for protein extraction or immunohistochemistry of frozen sections, and the other was fixed in 4% glutaraldehyde/paraformaldehyde for 30 minutes followed by 10% formalin overnight before being embedded in paraffin as previously described (25).

Syngeneic tumors were created with LKR-13, a lung adenocarcinoma cell line derived from a Kras<sup>A1</sup> mouse, in wild-type (129/sv) littermates of Kras<sup>A1</sup> mice as described (25). Briefly, 10<sup>7</sup> LKR-13 cells in 100 μL of PBS were s.c. injected into the flanks of the mice. When tumor volumes reached 50 mm<sup>3</sup>, they were randomly assigned to receive 500 μL of CIS (n = 5) or NGS (n = 5) by i.p. injection thrice a week. Tumor diameters were measured daily by an investigator (M.W.) who was blinded to the treatment group. Tumor volumes were calculated as 0.5 × × × diameter<sup>2</sup>. Treatment was continued until day 8, when one tumor reached 1,500 mm<sup>3</sup>, at which time all mice were killed. Tumors (n = 19) were then removed and either frozen at −80°C for protein extraction or fixed in 10% formalin overnight before being embedded in paraffin.

Cell lines. The LKR-13 and LKR-10 cell lines were derived by serial passaging of minced lung adenocarcinoma tissues isolated from a Kras<sup>A1</sup> mouse. The cells were passaged in RPMI 1640 supplemented with 10% fetal bovine serum on standard Falcon plasticware (Becton Dickinson, Bedford, MA) at 37°C in an atmosphere containing 5% CO<sub>2</sub>. The expression of the SV40 oncogenic protein was confirmed by Western blotting for the early and late antigens (27, 28). The cells were cultured in DMEM supplemented with 10% fetal bovine serum on standard plasticware at 33°C in an atmosphere containing 5% CO<sub>2</sub>. To generate conditioned medium, LKR-13 cells were plated in a 10-mm dish until 70% confluent, washed in PBS, and exposed overnight to 4 mL of RPMI without serum. The conditioned medium was recovered and centrifuged. The supernatant was recovered and frozen at −80°C for later use.

Bronchioalveolar lavage for isolation of inflammatory cells and supernatants. Briefly, Kras<sup>A1</sup> mice and wild-type littermates were killed by cervical dislocation, and three 1-mL aliquots of PBS were injected directly into the trachea. The liquid was recovered by gentle aspiration and centrifuged. The supernatant was recovered and frozen at −80°C for later use. The total cell count was done in a Neubauer chamber (Reichert, Buffalo, NY), and differential cell counts were done on cytospin preparations stained with H&E. The cell concentrations obtained by bronchioalveolar lavage varied between samples (1-8 × 10<sup>4</sup> cells and 10-40 × 10<sup>4</sup> cells for 129/sv and Kras<sup>A1</sup> mice, respectively). Cell viability was >98%, as assessed by trypan blue exclusion. Alveolar cells were suspended in RPMI 1640 serum-free medium containing penicillin G (100 IU/mL), streptomycin (100 μg/mL), and amphotericin B (0.25 μg/mL; Life Technologies) for the inoculation experiments.

Micro-CT analysis. Mice were anesthetized, intubated by veterinary personnel, and connected to a SAR-830/P small-animal ventilator (CWE, Ardmore, PA) operating using LabView software (National Instruments, Austin, TX), which provided controlled repeated breath holds during CT data acquisition (30). A single respiratory-gated, three-dimensional micro-CT image set was acquired for each mouse using 80 kVp, 405 μA, 400 millisecond per view, 720 views, and 0.5 degree incrementation per view. This acquisition resulted in a set of contiguous axial DICOM-formatted images through each mouse thorax, with voxels of dimensions 91 × 91 × 91 μm<sup>3</sup>. Lesions were characterized on the initial and final scans by one investigator (M.W.) who was blinded to the treatment group and autopsy results. Lesions visualized on the CT images included solid or “ground-glass” opacities or areas of consolidation resembling adenocarcinoma with bronchioloalveolar features in humans (31).

Tissue microarrays. Microarrays were constructed with cores from formalin-fixed, paraffin-embedded blocks. One array was made with specimens of normal lung tissue (n = 30), AAH (n = 40), adenoma (n = 206), and adenocarcinoma (n = 11) from lungs of Kras<sup>A1</sup> mice according to the histologic criteria established by Johnson et al. (23). A second array comprised all lesions identified by histologic analysis from the mice treated with CIS or NGS (a total of 36 AAH and 103 adenomas). Tissue microarrays were analyzed with the guidelines of The University of Texas M.D. Anderson Cancer Technology Consultants Laboratory, Newburg, OR). Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI) II (Vysis, Downers Grove, IL). CIS and NGS were obtained as described previously (15). IgG fractions were purified from the sera with a protein G column (IgG purification kit; Pierce, Rockford, IL) and concentrated with a centrifugal filter (Millipore, Bedford, MA).

Antibodies. We purchased rabbit polyclonal antibodies against KC (R&D Systems, Minneapolis, MN), factor VIII (DakoCytomation, Carpinteria, CA), prosurfactant protein C (SPC; Research Diagnostics, Concord, MA), and cleaved caspase-3 (Cell Signaling Technology, Beverly, MA); rat monoclonal antibodies against F4/80 and the neutrophil antigen p40 (Serotec, Oxford, United Kingdom); and mouse monoclonal antibodies against vascular endothelial growth factor (VEGF; Santa Cruz Biotechnology, Santa Cruz, CA). For immunofluorescence staining, we used rabbit antibodies against cleaved caspase-3 (FITC conjugated; Cell Signaling Technology) and CD31 (PE conjugated; Research Diagnostics) and secondary anti-rabbit and anti-rat antibodies (TRITC conjugated; Immunology Consultants Laboratory, Newburg, OR). Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI) II (Vysis, Downers Grove, IL). CIS and NGS were obtained as described previously (15). IgG fractions were purified from the sera with a protein G column (IgG purification kit; Pierce, Rockford, IL) and concentrated with a centrifugal filter (Millipore, Bedford, MA).

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Immunochemistry analysis. Four-micrometer tissue sections were deparaffinized, rehydrated, and washed with PBS as described (26). Antigen retrieval was performed with 0.01 mol/L citrate buffer (pH 6; DakoCytomation) for 30 minutes in a steamer. To detect the macrophage antigen (32), we used rabbit antibodies against F4/80 and the neutrophil antigen p40 (Serotec, Oxford, United Kingdom); and mouse monoclonal antibodies against F4/80 and the neutrophil antigen p40 (Serotec, Oxford, United Kingdom). Sections were incubated with peroxidase/PBS, avidin/biotin solution (Zymed, San Francisco, CA), and antibodies against cleaved caspase-3 (FITC conjugated; Cell Signaling Technology) and CD31 (PE conjugated; Research Diagnostics). Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI) II (Vysis, Downers Grove, IL). CIS and NGS were obtained as described previously (15). IgG fractions were purified from the sera with a protein G column (IgG purification kit; Pierce, Rockford, IL) and concentrated with a centrifugal filter (Millipore, Bedford, MA).

CXCR2 Ligands in KRAS-Induced Lung Cancer
lung adenocarcinoma cells for the macrophage antigen F4/80, the neutrophil antigen p40, and factor VIII; and used the isotype control for CXCRL2. For positive controls, we used normal lung tissue from Kras\textsuperscript{LAI} mice (F4/80, p40, neutrophil antigen, and factor VIII) and lymphoma tissue from E\textsubscript{mu}-myc mice (cleaved caspase-3).

Staining was quantified independently by two investigators (M.W. and L.W.) who were blinded to the treatment group. Lesions were scored based on the frequency of stained cells within lesions. Tissues were visualized at $\times20$ magnification for scoring of all antigens except cleaved caspase-3, which was visualized at $\times40$ magnification.

**Immunofluorescence staining.** For dual-fluorescence staining, frozen sections 4 µm thick were fixed in acetone and incubated with a fluorochrome-coupled primary antibody or with a primary antibody followed by a fluorochrome-coupled secondary antibody. Immunofluorescence-generated signals were visualized with a Zeiss Axioplan epifluorescence microscope (Nikon, Melville, NY) equipped with an oil immersion objective and single bandpass filters for FITC, Texas red, and DAPI. Digitized images of each fluorochrome were captured individually using a high-resolution image analysis system (MetaMorph, Universal Imaging, Downingtown, PA) with a cooled charged-couple device camera (C4742-95-12; Hamamatsu Photonics, Hamamatsu, Japan).

**ELISA.** To measure concentrations of KC, MIP-2, VEGF, and CXCL8 in lung homogenates, bronchoalveolar lavage supernatants, and conditioned medium samples, ELISA was done according to the manufacturer's instructions (R&D Systems). Results were expressed as the mean concentration (pg/mL) ± SE normalized to the amount of total protein.

**Western blot analysis.** Lysates from cell lines and tissue samples were separated by SDS/PAGE and transferred onto a polyvinylidene fluoride nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). Membranes were immunoblotted overnight at 4°C with primary antibodies in TBS containing 5% nonfat dry milk. Antibody binding was detected with an enhanced chemiluminescence kit according to the manufacturer's instructions (Amersham, Arlington Heights, IL).

**Cytokine array.** Cytokine antibody arrays (Array 3.1, Ray Biotech, Norcross, GA) were treated for 30 minutes at room temperature with blocking buffer and then incubated overnight at 4°C with lysates (100 µg) from Kras\textsuperscript{LAI} mouse–derived lung tissues. Protein binding was detected using the detection buffers as recommended by the manufacturer. The membrane has controls for the detection reaction (immobilized antigen/antibody complexes) and antibody specificity (antibodies against unrelated peptides or the absence of antibodies). Antibodies against murine peptides are arranged in duplicate on the array.

**Proliferation assay.** LKR-13 cells and MECs were plated at 1,000 per well in 96-well tissue culture plates and incubated for 24 hours before being treated with different concentrations of recombinant KC and MIP-2 (0-25 ng/mL; R&D Systems) or CXCR2 antibodies from CIS or IgG from NGS (1:50 to 1:2,000). Proliferation was quantified after 3 and 6 days by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

**Migration assay.** Cells (1 $\times 10^5$) in 750 µL of RPMI with 0.1% bovine serum albumin (Sigma, St Louis, MO) were plated in the upper chambers of wells containing a polyethylene terephthalate filter with 8-µm pores (BD BioCoat, San Jose, CA). For the experiments on MECs and LKR-13 cells (Fig. 2A and C-H), four wells per condition (with or without chemottractant) were used in each experiment, and the experiments were repeated thrice. The data shown are an average of the three experiments (12 data points per condition). For experiments on primary inflammatory cells derived from lavage samples (Fig. 2B), each experiment included two lavage samples (one sample from two Kras\textsuperscript{LAI} mice). Cells recovered from each lavage sample were split into two wells, which were treated with or without chemotactractant (two wells per condition, a total of four wells). This experiment was repeated thrice, and the mean values were calculated from six data points per condition. In Fig. 2G, primary inflammatory cells were recovered from lavage samples from five Kras\textsuperscript{LAI} mice. Cells from each mouse were split into four wells, two wells per condition (IgG- or CXCR2-neutralizing antibody). The two values per condition were averaged. The results from each mouse were presented separately, and the results from all five mice were combined to determine statistical significance. For these experiments, RPMI alone, RPMI with recombinant KC and MIP-2 (0-25 ng/mL), LKR-13 cell-conditioned medium, or bronchoalveolar lavage supernatant (1/2 in RPMI) was used as a chemoattractant in the lower chamber. To measure sensitivity to CXCR2-neutralizing antibodies, the cells were exposed to CXCR2-neutralizing antibodies or IgG (1:50 to 1:20,000) purified from CIS and NGS, respectively, during the assay. After 24 hours of incubation at 37°C, the cells on the upper surface of the membrane were removed with a cotton swab, leaving the cells on the lower surface, which were stained with Wright stain in methanol and examined by microscopy at $\times20$ magnification. Five fields were counted per filter.

**Statistical analysis.** Immunostaining scores, lung lesion numbers, and results from the proliferation and migration assays were considered continuous variables. The Kruskal-Wallis test was used for three-group comparisons and the Mann-Whitney nonparametric test for two-group comparisons. Spearman's r coefficient was used for correlative studies between quantitative variables. Ps $\leq 0.05$ were considered significant. Data were processed with StatView and Survival Tools 5.0 software (Abacus Concepts, Berkeley, CA).

**Results.**

**Chemokine expression in the lungs of Kras\textsuperscript{LAI} mice.** We first investigated chemokine expression in lung extracts derived from Kras\textsuperscript{LAI} mice using an array of antibodies against a panel of 62 murine chemokines. We examined lung tissues from mice at an early stage of lung tumorigenesis (5 months old) when AAH and adenomas but no adenocarcinomas were present to identify chemokines that might play a role in tumorigenesis. The chemokines expressed most prominently were cutaneous T-cell–attracting chemokine/C-C chemokine-27, CXCL-16, insulin-like growth factor binding protein-3, IL-1x, lipopolysaccharide-induced CXC-chemokine, IL-12/p70, lymphotactin, monocyte chemotactractant protein-1 (MCP-1), MCP-5, macrophage colony stimulating factor, MIP-2, MIP-3a, platelet factor-4, P-selectin, thymus-derived chemotactic agent-3, and vascular cell adhesion molecule-1 (Supplementary Fig. S1). Thus, a variety of chemokines were present in lung tissues of Kras\textsuperscript{LAI} mice before the development of lung adenocarcinoma.

**Biological evidence of CXCR2 ligands in the lungs of Kras\textsuperscript{LAI} mice.** Given the recent report that CXCL-8 is a transcriptional target of mutant KRAS (19), we further examined the role of the MIP-2 and KC in Kras\textsuperscript{LAI} mice. We investigated biological evidence of these ligands in Kras\textsuperscript{LAI} mice by examining premalignant alveolar lesions for neutrophils and vascular endothelial cells, which are recruited by these CXCR2 ligands. A tissue microarray was constructed for immunohistochemical analysis with punch biopsy samples of normal tissue, AAH, adenoma, and adenocarcinoma from lungs of Kras\textsuperscript{LAI} mice. We examined the expression of a 40-kDa cell surface antigen expressed specifically in neutrophils (32) and factor VIII, which is expressed in endothelial cells. Staining for neutrophils (Fig. 1A) and endothelial cells (Fig. 1B) in neoplastic lesions increased with histologic progression from AAH to adenocarcinoma. Cytospin preparations of bronchoalveolar lavage samples revealed that macrophages were the predominant inflammatory cell in the lavage samples from Kras\textsuperscript{LAI} mice and 129/sv wild-type littermates (Table 1). Neutrophil counts were significantly higher in bronchoalveolar lavage samples from Kras\textsuperscript{LAI} mice than in those from wild-type littermates (Table 1). Thus, malignant progression was associated with neutrophil inflammation and angiogenesis.

We next investigated biological evidence of CXCR2 ligands in bronchoalveolar lavage samples from Kras\textsuperscript{LAI} mice and in conditioned media samples from LKR-13 lung adenocarcinoma.
cells derived from Kras\textsuperscript{LA1} mice. The ability of these samples to chemoattract MECs, primary cultures of alveolar inflammatory cells, and LKR-13 cells was measured \textit{in vitro}, and the specific role of CXCR2 was examined by preincubation of the cells with a CXCR2-neutralizing antibody or IgG as a control (purified from CIS and NGS, respectively) to block cellular migration upon exposure to chemoattractants. Incubation with LKR-13 cell–conditioned medium recruited MECs (Fig. 2A) and alveolar inflammatory cells (Fig. 2B), whereas incubation with bronchoalveolar lavage supernatants recruited MECs (Fig. 2C) and LKR-13 cells (Fig. 2D). Preincubation with CXCR2-neutralizing antibody blocked the recruitment of MECs (Fig. 2E) and LKR-13 cells (Fig. 2F) by bronchoalveolar lavage supernatants and the recruitment of alveolar inflammatory cells (Fig. 2G) by LKR-13 cell–conditioned media samples, showing biological evidence of CXCR2 ligands in Kras\textsuperscript{LA1} mouse–derived lung tissues that was required for the recruitment of these cells. Preincubation with the CXCR2-neutralizing antibody did not block the recruitment of MECs by LKR-13 cell–conditioned medium (Fig. 2H), suggesting that LKR-13 cells secrete factors other than CXCR2 ligands that recruit MECs. Thus, lung tissues derived from Kras\textsuperscript{LA1} mice expressed soluble, biologically active mediators that recruited inflammatory cells, vascular endothelial cells, and adenocarcinoma cells, and this recruitment was inhibited, in part, by a CXCR2-neutralizing antibody.

**Table 1.** Inflammatory cells in bronchoalveolar lavage samples

<table>
<thead>
<tr>
<th>Mice</th>
<th>Total cells</th>
<th>Alveolar macrophages</th>
<th>Neutrophils</th>
<th>Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. cells/μL (% ± SE total cells), mean ± SE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type (n = 3)</td>
<td>106.6 ± 52.3</td>
<td>99.3 ± 47.7 (94.3 ± 1.2)</td>
<td>1.7 ± 1.3 (2.7 ± 1.6)</td>
<td>5.5 ± 5.3 (2.9 ± 2.1)</td>
</tr>
<tr>
<td>Kras\textsuperscript{LA1} (n = 4)</td>
<td>180.0 ± 58.4</td>
<td>136.4 ± 41.7 (79.6 ± 2.6)</td>
<td>25.7 ± 9.3 (18.3 ± 1.4)</td>
<td>9.3 ± 4.2 (46 ± 1.4)</td>
</tr>
<tr>
<td>( P^* )</td>
<td>0.7</td>
<td>0.4 (0.03)</td>
<td>0.03 (0.03)</td>
<td>0.28 (0.7)</td>
</tr>
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*Statistical analysis was done using the Mann-Whitney nonparametric test.
High expression of KC and MIP-2 in KrasLA1 mice. We examined expression of KC and MIP-2, the functional homologues of CXCL8 in mice, in lung tissues derived from KrasLA1 mice and their wild-type littermates by immunohistochemical analysis and ELISA. KC was detected by immunohistochemical analysis in epithelial cells, macrophages, and endothelial cells within alveolar epithelial lesions of KrasLA1 mice (Fig. 3A), whereas MIP-2 was not detectable by immunohistochemical analysis (data not shown). ELISA done on lung homogenates and supernatants from bronchoalveolar lavage samples showed that KrasLA1 mice had higher expression of KC (Fig. 3B) and MIP-2 (Fig. 3C) than did their wild-type littermates. The concentrations of KC and MIP-2 were highly correlated in lung homogenates (r = 0.92) and bronchoalveolar lavage fluid (r = 0.89; Fig. 3D), suggesting coordinated regulation of KC and MIP-2 expression in alveolar neoplastic lesions. In contrast, the expression of another angiogenic factor (VEGF) was not increased in the lungs of KrasLA1 mice (Fig. 3E and F), suggesting a specific role for CXCR2 ligands as angiogenic factors in early neoplastic lesions.

To extend the analysis to human lung cells, we investigated whether the expression of CXCL8 in HBECs, which are human bronchial epithelial cells that have been immortalized by the introduction of genes encoding cyclin-dependent kinase-4 and human telomerase reverse transcriptase (29), is increased by oncogenic KRAS. KRAS/HBECs, which have been transfected with a retroviral vector expressing mutant KRAS, acquire enhanced anchorage-independent growth and increased saturation density.9 CXCR2 expression was similar in HBECs and KRAS/HBECs (Fig. 4A), whereas CXCL8 expression, as measured by ELISA of conditioned media, was higher in KRAS/HBECs than in HBECs (Fig. 4B). Thus, the introduction of mutant KRAS was sufficient to confer high CXCL8 expression in HBECs.

CXCR2 neutralization inhibits alveolar epithelial neoplasia in KrasLA1 mice. We examined the role of CXCR2 in KrasLA1 lung tumorigenesis by passively immunizing mice with CIS or NGS (as a control). Beforehand, we examined CXCR2 expression in premalignant alveolar lesions by immunohistochemical analysis and found that CXCR2 was expressed in epithelial cells, inflammatory cells, and endothelial cells (Fig. 5A), indicating that multiple cell types were potential targets of CXCR2 neutralization. KrasLA1 mice were treated with CIS or NGS by i.p. injection thrice a week for 3 weeks, beginning at 4 months of age. After treatment, we counted the lesions on lung pleural surfaces at the time of autopsy. The mice were also subjected to micro-CT at the beginning and

9 J.D. Minna, unpublished data.
completion of treatment to examine changes in lesion numbers (examples are shown in Fig. 5C). Histologic analysis of lung tissue sections showed that the CIS-treated mice had fewer adenomas per mouse (mean ± SE: 3.3 ± 0.7 versus 6.0 ± 0.8; P = 0.01) and more AAH lesions (2.3 ± 0.4 versus 0.9 ± 0.2; P = 0.01) than did the NGS-treated mice. Together, these data suggest that CXCR2 neutralization decreased the number of lung adenocarcinoma precursors and inhibited the progression of AAH to more advanced stages of alveolar malignancy.

**CXCR2 neutralization induces apoptosis of vascular endothelial cells.** Based on the decrease in number of alveolar lesions observed after CIS treatment, we examined whether CXCR2 inhibition induces apoptosis. Western blot analysis showed that caspase-3 cleavage was higher in whole-lung extracts from mice treated with CIS than in those from mice treated with NGS (n = 6 mice per group; Fig. 6A), suggesting the presence of apoptotic cells. We also examined caspase-3 cleavage specifically in alveolar lesions. A tissue microarray for immunohistochemical analysis was constructed with punch biopsy samples of normal tissue, AAH, and adenoma from lungs of Kras<sup>LA1</sup> mice treated with CIS or NGS. Staining for cleaved caspase-3 was greater in alveolar lesions from mice treated with CIS than in those from mice treated with NGS (Fig. 6B).

We next investigated which cell types underwent apoptosis by performing dual immunofluorescence staining for cleaved caspase-3 and CD3 (endothelial cells), F4/80 (macrophages), p40 (neutrophils), or SPC (type II alveolar cells). We chose SPC as an epithelial cell marker because it is expressed in lung adenocarcinomas in Kras<sup>LA1</sup> mice (22). We observed coexpression of cleaved caspase-3 with CD3 (Fig. 6C) but not with F4/80, p40, or SPC (data not shown), suggesting that CXCR2 inhibition induced apoptosis of endothelial cells but not inflammatory cells or transformed alveolar epithelial cells.

**Sensitivity to CXCR2 inhibition requires the tumor microenvironment.** From the above findings, we could not exclude the possibility that CXCR2 neutralization inhibited lung tumorigenesis through effects on the tumor microenvironment, epithelial cells, or both. To investigate this question, we compared the *in vitro* and *in vivo* sensitivity of LKR-13 cells to CXCR2 neutralization. Despite their high expression of CXCR2 and KC (Fig. 7A), LKR-13 cells showed no change in proliferation after 5 days of treatment *in vitro* with CXCR2-neutralizing antibody (data not shown). To investigate effects of CXCR2 neutralization on LKR-13 cells *in vivo*, we established LKR-13 cells as syngeneic tumors in wild-type

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**Figure 3.** Alveolar lesions in Kras<sup>LA1</sup> mice have high expression of KC and MIP-2.

A, immunohistochemical analysis of KC in tumor cells (left; magnification, ×20) and macrophages (right; magnification, ×40) of an adenoma from a Kras<sup>LA1</sup> mouse. Arrows, positively stained cells. ELISA was used to detect KC (B), MIP-2 (C), and VEGF (E) in lung homogenates and bronchoalveolar lavage supernatants (BAL) from Kras<sup>LA1</sup> mice (n = 9) and wild-type (WT) littermates (n = 6). The concentrations of cytokines were normalized to the total protein content of each sample. Each ELISA was done in duplicate. Columns, means; bars, SE. D, KC and MIP-2 concentrations were correlated in each sample (●) of lung homogenate and bronchoalveolar lavage supernatant. F, immunohistochemical analysis of VEGF (magnification, 20×) shows staining in normal bronchial cells (left) and faint staining in tumor cells (right). Arrows, positively stained cells.
littermates. Once established as syngeneic tumors, LKR-13 cells recruited neutrophils and endothelial cells (Fig. 7B). Treatment of the mice with immune sera (CIS or NGS) every other day revealed that after 8 days, tumor volumes were lower in CIS-treated mice than in NGS-treated control mice ($P < 0.05$; Fig. 7C). Thus, the tumor microenvironment was required for the antitumor effect of CXCR2 inhibition on LKR-13 cells.

**Discussion**

This study provides evidence that alveolar epithelial cells transformed by oncogenic KRAS have high expression of CXCR2 ligands, which recruit inflammatory and endothelial cells, creating a milieu that promotes the progression of early neoplasia. Our finding that oncogenic KRAS triggers tumor-host interactions that are crucial to the promotion of tumor growth provides a rationale to target CXCR2 or its ligand, CXCL8, in the treatment of oncogenic KRAS-induced lung malignancy. If such a treatment approach is found to be efficacious, the implications are considerable given that there is currently no effective treatment for this subset of NSCLC.

Introduction of mutant RAS into cells is sufficient to confer transformed properties *in vitro* (19). In addition to these cell-autonomous effects, Ras promotes tumorigenesis *in vivo* by eliciting a stromal response, an effect mediated, in part, through CXCR2 ligands (19), which we showed here to have relevance to lung cancer. Mutant RAS increases the expression of CXCL8 through activation of Rac- and Akt-dependent pathways (19). We previously showed that Rac- and Akt-dependent pathways are activated in premalignant alveolar lesions of KrasA1 mice (33, 34), and that these pathways cooperate to maintain the survival of lung cancer cells (35). Rac- and Akt-dependent pathways promote cell survival by regulating the expression of BCL2 family members, caspase-9, FOXO proteins, and GSK-3, among others (36–38). Thus, oncogenic RAS induces cellular transformation through cell-autonomous and stroma-dependent processes, both of which are activated through Rac- and Akt-dependent pathways, which cooperate to modulate the expression of a diverse set of downstream mediators.

Findings reported here on the role of CXCR2 ligands in inflammation and neovascularization corroborate evidence from other pulmonary diseases in which these processes are prominent factors. In bronchiolitis obliterans syndrome, which occurs after lung transplantation, and in the adult respiratory distress syndrome, CXCR2 ligands have been shown to play a role. In both conditions, CXCR2 ligands are upregulated in response to inflammation, and inhibition of CXCR2 has been shown to have beneficial effects in animal models (20). These findings suggest that targeting CXCR2 may be a promising therapeutic approach for the treatment of lung cancer and other pulmonary diseases.

**Figure 4.** Mutant KRAS increases CXCL8 expression in HBECs. A, Western blot analysis of HBECs and KRAS/HBECs using specific antibodies against CXCR2 and β-actin. B, ELISA for CXCL8 in conditioned medium collected from HBECs and KRAS/HBECs at various times after seeding. Normalized to total protein content.

**Figure 5.** CXCR2 neutralization inhibits malignant progression in KrasA1 mice. A, Immunohistochemical analysis of CXCR2 in tumor cells (top; magnification, ×20) and endothelial cells (bottom; magnification, ×40) in a lung adenoma from a KrasA1 mouse. Arrows, positively stained cells. B, Representative micro-CT axial images of one KrasA1 mouse at the beginning (top) and end (bottom) of treatment with CXCR2-neutralizing antibody. The lesions, which appear as peripheral nodules in the right and left lower lobes (arrows), disappeared with treatment; the other lesions decreased moderately or were stable. C, Mean numbers of lesions per mouse were determined for each group (mice treated with CXCR2 antibody (CIS) or NGS) by counting visible lesions on the pleural surfaces at autopsy (left). Changes in lesion numbers over time were determined from micro-CT scans done at the beginning and end of treatment (right). Columns, means; bars, SE.
syndrome caused by ventilator-induced lung injury, lung expression of CXCR2 ligands parallels the inflammatory response and lung injury (17, 39). Neutralization of CXCR2 is sufficient to attenuate neutrophil infiltration and lung injury in murine models of these diseases (17, 39). In the heterotopic and orthotopic Lewis lung cancer models, tumor growth is associated with enhanced neovascularization, neutrophil inflammation, and expression of CXCR2 ligands, and CXCR2 neutralization decreases tumor size and increases tumor necrosis (15). Similar to our findings with LKR-13 cells, the CXCR2-neutralizing antibody is efficacious against Lewis lung cancer cells grown as heterotopic tumors but has no effect on their proliferation in vitro, indicating that the tumor microenvironment is required for tumor sensitivity. Thus, high expression of CXCR2 ligands contributes to inflammation and neovascularization in a broad spectrum of pulmonary diseases.

Our observation that CXCR2 ligands contribute to the establishment and growth of tumor cells in a syngeneic host raises the possibility that they can also promote the progression of early neoplasia toward invasive disease. In Kras^{L5} mice, in which the expansion of hyperplastic alveolar epithelial cells into adenomas precedes the development of adenocarcinomas (23), we found that malignant progression was associated with enhanced neutrophil inflammation and neovascularization and high expression of CXCR2 ligands. CXCR2 neutralization reduced the numbers of early lesions, inhibited the progression of AAH into adenomas, and induced apoptosis of vascular endothelial cells. These findings suggest that high expression of CXCR2 ligands and the accompanying neovascularization are required for early alveolar neoplasia. The angiogenic activity of CXCR2 ligands has been reported to be neutrophil-dependent in the Matrigel sponge angiogenesis assay (40). Although we have not excluded a role for neutrophils in angiogenesis in Kras^{L5} mice, CXCR2 neutralization did not decrease the numbers of neutrophils in premalignant alveolar lesions by immunohistochemical analysis of lung tissue sections (data not shown), raising the possibility that CXCR2 ligands promote angiogenesis through direct effects on vascular endothelial cells. Based on the response of this early neoplastic disease model to CXCR2 inhibition, strategies to target CXCR2 may find application in treating early lung cancers in humans.

Oncogenic KRAS has been reported to increase the expression of VEGF in a variety of tumor types, including lung cancer (41, 42). Based on these and other findings, clinical strategies have used neutralizing antibodies to VEGF and VEGF receptor, VEGF ligand traps, and small-molecule inhibitors of VEGF receptor tyrosine kinase activity to block neovascularization in patients with malignancies. However, a recent study found high CXCL8 expression in oncogenic KRAS-transformed colon cancer cells deficient in hypoxia-inducible factor-1 and VEGF, and CXCL8 was sufficient to promote angiogenesis in that setting (43). Thus, CXCL8 expression contributes to angiogenesis in KRAS-transformed cells and increases in response to VEGF blockade.
raising the possibility that treatment of KRAS-mutant tumors with antiangiogenic strategies targeting both CXCL8 and VEGF may be more effective than VEGF blockade alone. Based on these preclinical studies, efforts are warranted to investigate CXCR2 as a therapeutic target for the prevention and treatment of NSCLC.

Figure 7. CXCR2 inhibition shrinks LKR-13 syngeneic tumors. A, left, Western blot analysis of LKR-10 and LKR-13 cells using specific antibodies against CXCR2 and β-actin. Middle, immunohistochemical analysis of CXCR2 in LKR-13 cells. Right, ELISA for MIP-2 and KC in conditioned medium from LKR-13 cells. B, immunohistochemical analysis of LKR-13 syngeneic tumors to identify infiltrating endothelial cells and neutrophils using specific antibodies against factor VIII (left) and p40 (right; magnification, ×40). C, changes in mean volumes of LKR-13 syngeneic tumors over time in mice treated with CXCR2 antibody (CIS) or NGS. Tumors were measured daily. Points, mean tumor volumes; bars, SE. *, P < 0.05.

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