Interleukin-6 Prevents the Initiation but Enhances the Progression of Lung Cancer
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Abstract
Recent studies suggest that high expression of the proinflammatory cytokine IL6 is associated with poor survival of lung cancer patients. Accordingly, IL6 has been a target of great interest for lung cancer therapy. However, the role of IL6 in lung cancer has not been determined yet. Here, we demonstrate that IL6 plays opposite roles in the initiation and growth of lung cancer in a mouse model of lung cancer induced by the K-Ras oncogene. We find that compared with wild-type mice, IL6-deficient mice developed much more lung tumors after an activating mutant of K-Ras was induced in the lungs. However, lung tumors developed in IL6-deficient mice were significantly smaller. Notably, both the lung tumor-suppressing and -promoting functions of IL6 involve its ability in activating the transcription factor STAT3. IL6/STAT3 signaling suppressed lung cancer initiation through maintaining lung homeostasis, regulating lung macrophages, and activating cytotoxic CD8 T cells under K-Ras oncogenic stress, whereas it promoted lung cancer cell growth through inducing the cell proliferation regulator cyclin D1. These studies reveal a previously unexplored role of IL6/STAT3 signaling in maintaining lung homeostasis and suppressing lung cancer induction. These studies also significantly improve our understanding of lung cancer and provide a molecular basis for designing IL6/STAT3-targeted therapies for this deadliest human cancer. Cancer Res; 75(16); 3209-15. ©2015 AACR.

Introduction
Lung cancer is the leading cause of cancer deaths in both women and men, responsible for roughly 160,000 deaths annually in the United States alone (1). Moreover, approximately 85% of the patients with lung cancer die of the disease within 5 years (1). A better understanding of the mechanisms underlying lung cancer development and progression and therapy resistance is direly needed to design novel effective therapies for this deadliest cancer. The most predominant risk factor for lung cancer is tobacco smoking, which accounts for about 87% of lung cancer cases (2). Tobacco smoke induces genetic alterations, particularly activating mutations of the K-Ras oncogene, in lung epithelium to initiate and promote carcinogenesis (2).

One of the important functions of K-Ras activation is to induce expression of IL6, a pleiotropic proinflammatory cytokine that has been suggested to function as a lynchpin between inflammation and cancer in several cancers, such as colon and liver cancers (3, 4). Indeed, IL6 is expressed in over 50% of human lung cancer cell lines and primary tissues (5, 6). As a matter of fact, IL6 can be detected in serum, pleural fluids, bronchoalveolar lavage fluids (BALF), and breath condensate of patients with lung cancer (6–10). More importantly, high IL6 level in tumor tissue, serum, BALF, and breath condensate is associated with lung cancer progression, resistance to antitumor therapies, and poor survival of lung cancer patients (6–10). Moreover, high IL6 level is also associated with postoperative complication and postoperative recurrence of lung cancer (11–13). Mechanistic studies suggest that IL6 promotes lung cancer cell proliferation and migration through activation of the transcription factor STAT3 (4, 7, 14). In line with the role of IL6 in STAT3 activation, STAT3 has been found to be persistently activated in up to 65% of human lung cancers (4, 14). Also, the constitutive activation of STAT3 is associated with lung cancer progression, therapy resistance, and poor survival of lung cancer patients (4, 14). These studies suggest a molecular link between IL6 and lung cancer.

However, it remains unknown whether and how IL6 is involved in the initiation of lung cancer. Current studies on lung cancer mainly focus on the role of IL6 in the in vivo growth of cell culture and in vivo growth in immunodeficient mice of lung cancer cell lines (5, 15). Although useful, these studies require validation in endogenously arising lung tumors. They cannot address the role of IL6 in the early stages of lung tumorigenesis. Furthermore, they cannot determine whether and how the inflammation-regulatory activity of IL6 is involved in lung cancer, because the hosts they used for the in vivo growth of lung cancer cells lack immune responses and immunity. Another important issue that still remains to be determined is the role of IL6 in lung physiology under oncogenic stresses. Addressing these issues is of importance and interest, given the pleiotropic and complex functions of IL6. In particular, using endogenous lung tumorigenesis in immune-competent mice as a model system, we have recently found that STAT3 plays opposing roles in the initiation and progression of
lungs were examined (16). Accordingly, we also examined the effect of IL6 deficiency on the initiation and development of endogenous lung tumor in immune-competent mice.

**Materials and Methods**

**Animals**

IL6 knockout (IL6/Δ/Δ) mice were purchased from The Jackson Laboratory. Lox-Stop-Lox (LSL) K-RasG12D mice were described previously (16). Both IL6/Δ/Δ mice and LSL-K-RasG12D mice were backcrossed to FVB/N mice for more than ten generations for pure FVB/N background. IL6/Δ/Δ FVB/N mice and LSL-K-RasG12D FVB/N mice were then bred to generate IL6/Δ/Δ/LSL-K-RasG12D FVB/N mice. All animals were housed under specific pathogen-free conditions, and all animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

**Lung carcinogenesis and tumor enumeration**

Six- to 8-week-old IL6/Δ/Δ/LSL-K-RasG12D mice and IL6+/+/LSL-K-RasG12D mice were intranasally administered 1 × 10^3 plaque-forming units (pfu) of Cre-expressing adenovirus (adenocare; Gene Transfer Vector Core, University of Iowa, Iowa City, IA) to induce expression of the K-RasG12D mutation in lungs. Three months after Cre induction of K-RasG12D, all mice were sacrificed for lung tumor examinations. Surface tumors in mouse lungs were counted by three blinded readers under a dissecting microscope. Tumor diameters were determined by microcalipers.

**BALF and immunofluorescence assays**

Mice were sacrificed, and their lungs were lavaged four times with PBS. The recovered BALF were centrifuged. Cells from BALF were visualized by Hema 3 staining, and different leukocytes were counted. Cells from BALF were also subjected to immunofluorescence assays as described previously (16). The antibodies used for immunofluorescence staining were listed in Supplementary Table S1.

**IHC assays**

Mouse lungs were excised, fixed in formalin, embedded in paraffin, and cut into 4-μm-thick sections. Sections were subjected to IHC staining as described previously (16). The antibodies used for IHC staining were listed in Supplementary Table S1.

**BrdUrd labeling**

Mice were i.p. injected with 50 mg/kg BrdUrd (Sigma-Aldrich) 24 hours prior to sacrifice. Mouse lung tissue sections were stained with anti-BrdUrd (Sigma-Aldrich) according to the vendor’s instructions.

**Real-time PCR analysis**

Mouse lung tissues, lung tumor tissues, BAL cells, or lung epithelial cells were subjected to RNA extraction, RNA reverse transcription, and real-time PCR as described previously (16). Primer pairs used for real-time PCR were listed in the Supplementary Table S2.

**Statistical analysis**

Data were reported as mean ± SD. The Student t test (two-tailed) was used to assess significance of differences between two groups, and P values < 0.05 and 0.01 were considered statistically significant and highly statistically significant, respectively (16).

**Results**

**IL6/Δ/Δ mice are prone to lung tumorigenesis induced by mutant K-Ras**

To test the functional significance of IL6 in lung tumorigenesis, we took advantage of IL6/Δ/Δ mice and LSL-K-RasG12D mice. After the mutant K-RasG12D transgene is activated in lungs through intranasal administration of Cre recombinase, LSL-K-RasG12D mice develop lung cancers. It is worthy to note that murine lung cancers driven by oncogenic K-Ras faithfully recapitulate human lung cancers, and in particular adenocarcinomas associated with tobacco smoking (16). They share the same genetic and molecular changes, as well as morphology and histology. Moreover, K-Ras–induced lung cancers in mice, like their human counterparts, are associated with pulmonary damage and immune cell infiltration. Thus, we generated IL6/Δ/Δ/LSL-K-RasG12D mice and IL6+/+/LSL-K-RasG12D mice by breeding IL6/Δ/Δ mice and LSL-K-RasG12D mice. For simplicity, IL6/Δ/Δ/LSL-K-RasG12D mice and IL6+/+/LSL-K-RasG12D mice are hereinafter referred to as IL6/Δ/Δ mice and IL6+/+/ (or simply as wild type, WT) mice, respectively.

Consistent with previous studies (16), WT mice developed lung tumors after Cre induction of K-RasG12D in the lungs as evidenced by both the surface tumor enumeration and histologic assays (Fig. 1A–C). However, IL6/Δ/Δ mice developed more lung tumors after the same induction of K-RasG12D. Except for their difference in IL6 expression, tumors in IL6/Δ/Δ mice and WT mice had the same morphologic and histologic features (Fig. 1C and D). In further support of the notion that SP-C–positive alveolar type II epithelial cells and/or BASCs are the cells-of-origin of lung cancers (16), tumors in IL6/Δ/Δ mice or WT mice were positive for SP-C, while negative for the Clara cell marker CCSP (Clara cell secretory protein; Fig. 1C). These data indicated that IL6 suppresses lung tumor initiation induced by K-Ras.

**Increased lung tumorigenesis in IL6/Δ/Δ mice is associated with exacerbated lung damage as well as increased number and toxicity of lung macrophages**

Before K-RasG12D induction, the lungs of IL6/Δ/Δ mice were normal and displayed the same morphology and histology as those of WT mice (data not shown). In agreement with our recent findings (16), K-RasG12D expression in the lungs of WT mice induced mild alveolar congestion and minor impairments of alveolar epithelial integrity, indicating mild lung damage (Fig. 2A, left, black arrowheads). However, the same K-RasG12D expression caused more significant lung damage in IL6/Δ/Δ mice, as evidenced by the severe loss of the integrity of the alveolar epithelium and the enlarged air space in the lungs of IL6/Δ/Δ mice (Fig. 2A, right, black arrowheads). These data suggested that IL6 is required for maintaining pulmonary homeostasis under K-Ras oncogenic stress, including pulmonary inflammation induced by K-Ras (see the following sections).

To determine the mechanisms by which IL6 suppresses K-Ras–induced lung damage, we compared the activation status of STAT3 in the lung epithelial cells of IL6/Δ/Δ mice and WT mice. It has been well established that one of the most important functions of IL6 is to activate STAT3. Most
importantly, our recent studies show that lung epithelial STAT3 is indispensable for lung homeostasis under oncogenic stresses, including those induced by K-Ras activation or the tobacco carcinogen urethane (16). We found that compared with those in WT mice, lung epithelial cells in IL6^D/- mice were with much weaker nuclear staining of STAT3, suggesting a decreased STAT3 activation in these lung epithelial cells in IL6^D/- mice (Fig. 2A, empty arrows). These data together suggested that IL6 protects lungs from K-Ras–induced injury through activation of STAT3 intrinsic to lung epithelial cells.

We also examined the effect of IL6 deletion on lung macrophages, because macrophages are the most abundant immune cells in the lungs and have been linked to lung injury under several pathogenic conditions. Moreover, one of the best-known functions of IL6 is to regulate immune cells. Like lung epithelial cells, macrophages in the lungs of IL6^D/- mice also showed a significantly decreased STAT3 activation (Fig. 2A, filled black arrows). Interestingly, however, significantly more lung macrophages were detected in the lung tissues and BALF of IL6^D/- mice (Fig. 2A and B). Consistently, the monocyte-attractive chemokines CCL3 (also known as macrophage inflammatory protein-1 alpha, MIP-1α) and CXCL2 (also called macrophage inflammatory protein-2 alpha, MIP-2α) were significantly increased in the lung tissues of IL6^D/- mice (Fig. 2C). Another monocyte-attractive chemokine CXCL1 was also increased, although not statistically significant, in the lung tissues of IL6^D/- mice. The increase in the expression of CCL3, CXCL1, and CXCL2 was somewhat specific, as the expressions of many other cytokines and chemokines were comparable in the lungs or BALF of IL6^D/- mice and WT mice (Supplementary Fig. S1).

Notably, in comparison with lung macrophages in WT mice, lung macrophages in IL6^D/- mice expressed a higher level of nitric oxide synthase (iNOS), a potent inducer of cell damage (Fig. 2D). In line with in vivo data, addition of IL6 prevented iNOS induction in macrophages in vitro (Supplementary Fig. S2). These data suggested that IL6 suppresses iNOS expression in lung macrophages. In contrast, lung macrophages in IL6^D/- mice almost completely lost the ability to express IL10, although macrophages are the primary source of this anti-inflammatory cytokine (Fig. 2D). Nevertheless, these data are highly consistent with the finding that lung macrophages in IL6^D/- mice are defective in STAT3 activation and with the fact

Figure 1.
Increased lung tumorigenesis in IL6^D/- mice after K-RasG12D induction in lungs. A, lung tissues from IL6^D/- mice and WT mice. Representative tumors are indicated by arrows. B, increased lung tumor multiplicities in IL6^D/- mice. Data, mean ± SD (n ≥ 9; *, P < 0.05). C, histologic analysis showing increased adenomatous hyperplasia and tumor lesions in the lungs of IL6^D/- mice. Representative lesions are indicated by arrows. Data, mean ± SD (n ≥ 9; **, P < 0.01). Scale bar, 200 μm. D, IHC analysis of IL6, SP-C, and CCSP in lung tumors from IL6^D/- mice and WT mice. Scale bar, 50 μm.
that IL10 is a transcriptional target of STAT3. Interestingly, IL6 and IL10 induced each other in both macrophages and lung epithelial cells (Supplementary Fig. S3A and S3B), suggesting a paracrine loop of IL6/IL10. More importantly, IL6 and IL10 suppressed apoptosis of lung epithelial cells in vitro, which was associated with STAT3 activation and induction of cell survival genes, such as survivin, Bcl-2, and Bcl-xL (Supplementary Fig. S3C–S3E). Given the abundant expression of IL10 receptor (IL-10R) in lung epithelial cells, these data together suggested that IL6 and IL10 form a paracrine loop among macrophages and lung epithelial cells to activate STAT3, protecting lung epithelial cells from K-Ras–induced injury.

Increased lung tumorigenesis in IL6\(^{/A}\) mice is also associated with the decreased expansion and activation of CD8 T cells as well as the decreased tumor killing.

Another important role of IL10 is to activate and expand CD8 T cells, the lymphocytes that can directly induce apoptosis of tumor cells for tumor suppression. Thus, we hypothesized that IL10-mediated activation and expansion of cytotoxic CD8 T cells is another mechanism by which IL6 suppresses lung tumorigenesis induced by K-Ras. To test the hypothesis, we first examined the total numbers of T cells in the BALF of IL6\(^{/A}\) and WT mice. We found that compared with WT mice, IL6\(^{/A}\) mice had significantly fewer T cells in the BALF (Fig. 3A). The decrease of T cells in the lungs of IL6\(^{/A}\) mice was due to the loss of CD8 T cells, because the expression of CD8, but not that of CD4, was much lower in the BALF of IL6\(^{/A}\) mice (Fig. 3A). Notably, CD8 T cells in the lungs of IL6\(^{/A}\) mice had defective tumor-killing ability. Compared with lung CD8 T cells in WT mice, lung CD8 T cells in IL6\(^{/A}\) mice expressed much lower levels of antitumor cytokine IFN\(\gamma\) and apoptosis inducers granzyme A and granzyme B (Fig. 3B). Accordingly, lung tumor cells in IL6\(^{/A}\) mice had much lower apoptosis rate (Fig. 3C). It seems that the defects of the lung CD8 T cells in IL6\(^{/A}\) mice were largely due to their defect in STAT3 activation (Fig. 3D).

To confirm the in vivo data in a simple and direct way, we compared the in vitro tumor cell killing ability of CD8 T cells...
isolated from IL6\(^{-/-}\) mice and WT mice. As expected, coculture with CD8 T cells from WT mice led to loss of lung tumor cells (Supplementary Fig. S4A). However, CD8 T cells from IL6\(^{-/-}\) mice significantly lost the tumor killing ability. On the other hand, addition of IL6 significantly enhanced the tumor killing ability of CD8 T cells (Supplementary Fig. S4B). Consistently, we found that addition of IL6 increased expression of cytotoxic molecules in CD8 cells, such as perforin, granzymes A and B, TNF\(\alpha\), and TRAIL (Supplementary Fig. S4C). Moreover, IL6 also induced expression of cell survival genes in CD8 cells, such as Bcl-2, Bcl-xL, survivin, and Mcl-1, and prevented activation-induced death of CD8 cells in vitro (Supplementary Fig. S4D). These data altogether clearly indicated that IL6 also protects and activates cytotoxic CD8 T cells to suppress K-Ras-induced lung tumorigenesis.

**IL6 contributes to the growth of lung cancers induced by K-Ras.**

Although IL6\(^{-/-}\) mice developed more lung tumors than WT mice after K-Ras\(^{G12D}\) induction in lungs, the average sizes of tumors in IL6\(^{-/-}\) mice were significantly smaller (Fig. 4A). These data suggested that IL6 suppresses the initiation but paradoxically contributes to the growth of lung tumor induced by K-Ras.

To investigate the mechanisms by which IL6 contributes to lung cancer growth, we first compared the proliferation rates of lung tumors in IL6\(^{-/-}\) mice and WT mice using the BrdUrd cell proliferation assay. As shown in Fig. 4B, much fewer BrdUrd-positive cells were detected in the tumors from IL6\(^{-/-}\) mice compared with those from WT mice, indicating that the tumors in IL6\(^{-/-}\) mice have a lower proliferation rate. We then compared the STAT3 activation status in these lung tumors in IL6\(^{-/-}\) mice and WT mice. We found that STAT3 activation was significantly lower in the lungs from IL6\(^{-/-}\) mice (Fig. 4C). Consistently, cyclin D1, a downstream target gene of STAT3 that is well known for its critical roles in promoting cell proliferation, was significantly decreased in the lungs from IL6\(^{-/-}\) mice (Fig. 4D). These data suggested that IL6 contributes to the growth of lung tumors induced by K-Ras, most likely through activating STAT3 to induce expression of cell proliferation genes, such as cyclin D1. In further support of this, IL6 induced STAT3 activation and cyclin D1 expression and promoted growth of lung tumor cells in vitro (Supplementary Fig. S5). Moreover, our recent studies demonstrate that genetic deletion of STAT3 from lung tumors induced by K-Ras or urethane suppresses cyclin D1 expression and inhibits tumor growth in mice (16). Altogether, these data suggested that the IL6/STAT3 signaling plays opposite roles in the initiation and growth of lung cancer.

**Discussion**

IL6 is a target of great interest for the prevention and treatment of human lung and other cancers (18), concomitant with an appreciation for the critical role of IL6 in cancer cell growth and the association of high IL6 expression with cancer progression and poor survival of cancer patients (4). In addition, the enthusiasm also comes from clinical trial studies showing that IL6-based therapies are particularly effective and tolerably safe for several inflammation diseases, such as rheumatoid arthritis, systemic juvenile idiopathic arthritis, and Castleman's disease (19). Our findings identify a complex role for IL6 in lung cancer, preventing tumor induction while enhancing tumor growth (Figs. 1 and 4). In line with our findings, recent studies indicate that high IL6 expression is not
associated with lung cancer risk in humans (20), although it is associated with lung cancer progression and poor survival of lung cancer patients (6–10). It should be pointed out that even complete deletion of IL6 can only delay lung cancer growth. Thus, to target IL6 for lung cancer therapy, we need, on one hand, to consider the potential risk in increasing lung damage and tumorigenesis due to long-term IL6 inhibition, and on the other hand, to combine IL6 inhibition with other cancer therapies for efficient clinical outcomes. In this regard, recent phase I and II clinical trials involving 125 lung cancer patients indicate that IL6-targeted therapy alone has no obvious clinical benefits, except for an amelioration of lung cancer–associated anemia and cachexia in patients (19). Although more careful and more lung cancer patients–involved clinical trials are needed to determine the clinical outcomes of IL6-targeted therapy, it could be speculated that the overall clinical benefits of IL6 therapy alone might be limited, giving both the tumor-suppressing and -promoting roles of IL6 in lung cancer.

Interestingly, both lung tumor–suppressing and –promoting functions of IL6 involve its ability in activating STAT3. IL6 suppresses lung cancer induction through maintaining lung homeostasis and inducing tumor cell killing in STAT3-dependent manners. In addition to inducing STAT3 activation in lung epithelial cells, IL6 activates STAT3 in other cells in lungs, particularly macrophages, to express IL10, which serves as a paracrine stimulus to further enhance lung epithelial STAT3 activation for lung homeostasis under oncogenic stress (Fig. 2 and Supplementary Fig. S3). IL6 also suppresses lung macrophages to express iNOS and thereby prevents iNOS-induced lung damage (Fig. 2 and Supplementary Fig. S2). IL10 produced by lung macrophages, perhaps together with IL6, also induces STAT3 activation in cytotoxic CD8 T cells for their survival and activation, which in turn induce tumor cell apoptosis (Fig. 3 and Supplementary Fig. S4). Thus, it seems that IL6 utilizes multiple related mechanisms to suppress lung cancer initiation. Paradoxically, IL6 contributes to lung cancer growth also through STAT3-dependent mechanism. In this case, IL6 activates STAT3 to induce cyclin D1 in lung cancer cells, therefore promoting cancer proliferation (Fig. 4 and Supplementary Fig. S5). In further support of these findings, our recent studies indicate that mice selectively deficient in lung epithelial STAT3 show overall similar phenotypes as IL6-deficient mice in K-Ras–induced lung damage, tumor initiation, and progression (16).

In summary, these data demonstrate that the IL6/STAT3 signaling plays overall opposite roles in the initiation and growth of lung cancer—it prevents lung cancer initiation through maintaining lung homeostasis and inducing cytotoxic CD8 T cells, whereas it contributes to (although it is not absolutely required for) lung cancer growth through inducing expression of key regulators of cell proliferation. These studies not only greatly improve our understanding of the pathophysiologic actions of IL6/STAT3 signaling and the pathogenesis of lung and other cancers associated with IL6/STAT3 signaling, but also provide a mechanistic basis for targeting IL6/STAT3 signaling to treat IL6- and STAT3-associated cancers.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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