Interleukin-6 prevents the initiation but enhances the progression of lung cancer

Running title: IL-6 in lung cancer

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Abstract

Recent studies suggest that high expression of the pro-inflammatory cytokine interleukin-6 (IL-6) is associated with poor survival of lung cancer patients. Accordingly, IL-6 has been a target of great interest for lung cancer therapy. However, the role of IL-6 in lung cancer has not been determined yet. Here, we demonstrate that IL-6 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 to wild type mice, IL-6 deficient mice developed much more lung tumors after an activating mutant of K-Ras was induced in the lungs. However, lung tumors developed in IL-6 deficient mice were significantly smaller. Notably, both the lung tumor-suppressing and -promoting functions of IL-6 involve its ability in activating the transcription factor STAT3. IL-6/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 IL-6/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 IL-6/STAT3-targeted therapies for this deadliest human cancer.
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 interleukin-6 (IL-6), a pleiotropic pro-inflammatory 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, IL-6 is expressed in over 50% of human lung cancer cell lines and primary tissues (5, 6). As a matter of fact, IL-6 can be detected in serum, pleural fluids, bronchioalveolar lavage fluids (BALF) and breath condensate of patients with lung cancer (6-10). More importantly, high IL-6 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 IL-6 level is also associated with postoperative complication and postoperative recurrence of lung cancer (11-13). Mechanistic studies suggest that IL-6 promotes lung cancer cell proliferation and migration through activation of the transcription factor Signal Transducer and Activator of Transcription 3 (STAT3) (4, 7, 14). In line with the role of IL-6 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 IL-6 and lung cancer.
However, it remains unknown whether and how IL-6 is involved in the initiation of lung cancer. Current studies on lung cancer mainly focus on the role of IL-6 in the *in vitro* growth in 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 IL-6 in the early stages of lung tumorigenesis. Furthermore, they cannot determine whether and how the inflammation-regulatory activity of IL-6 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 IL-6 in lung physiology under oncogenic stresses. Addressing these issues is of importance and interest, given the pleiotropic and complex functions of IL-6. 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 lung tumor (16). Accordingly, we also examined the effect of IL-6 deficiency on the initiation and development of endogenous lung tumor in immune-competent mice.

**Materials and Methods**

**Animals**

IL-6 knockout (IL-6ΔΔ) mice were purchased from Jackson Laboratory. Lox-Stop-Lox (LSL) K-RasG12D mice were described previously (16). Both IL-6ΔΔ mice and LSL-K-RasG12D mice were backcrossed to FVB/N mice for more than ten generations for pure FVB/N background. IL-6ΔΔ FVB/N mice and LSL-K-RasG12D FVB/N mice were then bred to generate IL-6ΔΔ/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 (IACUC) of the University of Pittsburgh.
Lung carcinogenesis and tumor enumeration

Six- to eight-week-old IL-6Δ/Δ/LSL-K-RasG12D mice and IL-6wt/wt/LSL-K-RasG12D mice were intranasally administered 1 × 10^7 plaque-forming units (pfu) of Cre-expressing adenovirus (adenocre; Gene Transfer Vector Core, University of Iowa, Iowa City, IA) to induce expression of the K-RasG12D mutant in lungs. Three months post 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 (IF) assays

Mice were sacrificed, and their lungs were lavaged four times with phosphate buffered saline (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 IF assays as described previously (17). The antibodies used for IF staining were listed in Supplemental Table 1.

Immunohistochemistry (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 Supplemental Table 1.

BrdU labeling

Mice were i.p. injected with 50 mg/kg BrdU (Sigma-Aldrich) 24 hours prior to sacrifice. Mouse lung tissue sections were stained with anti-BrdU (Sigma-Aldrich) according to the vendor's instructions.
**Real-time polymerase chain reaction (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 Supplemental Table 2.

**Statistical Analysis**

Data were reported as mean ± standard deviation (SD). The Student’s 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**

**IL-6\( ^{\Delta/\Delta} \) mice are prone to lung tumorigenesis induced by mutant K-Ras**

To test the functional significance of IL-6 in lung tumorigenesis, we took advantage of IL-6\( ^{\Delta/\Delta} \) mice and LSL-K-Ras\( ^{G12D} \) mice. After the mutant K-Ras\( ^{G12D} \) transgene is activated in lungs through intranasal administration of Cre recombinase, LSL-K-Ras\( ^{G12D} \) 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 IL-6\( ^{\Delta/\Delta} \)/LSL-K-Ras\( ^{G12D} \) mice and IL-6\( ^{wt/wt} \)/LSL-K-Ras\( ^{G12D} \) mice by breeding IL-6\( ^{\Delta/\Delta} \) mice and LSL-K-Ras\( ^{G12D} \) mice. For simplicity, IL-6\( ^{\Delta/\Delta} \)/LSL-K-Ras\( ^{G12D} \) mice and IL-6\( ^{wt/wt} \)/LSL-K-Ras\( ^{G12D} \) mice are hereinafter referred to as IL-6\( ^{\Delta/\Delta} \) mice and IL-6\( ^{wt/wt} \) (or simply as wild type, WT) mice, respectively.
Consistent with previous studies (16), WT mice developed lung tumors after Cre induction of K-Ras\textsuperscript{G12D} in the lungs as evidenced by both the surface tumor enumeration and histological assays (Figures 1A-1C). However, IL-6\textsuperscript{Δ/Δ} mice developed more lung tumors after the same induction of K-Ras\textsuperscript{G12D}. Except for their difference in IL-6 expression, tumors in IL-6\textsuperscript{Δ/Δ} mice and WT mice had the same morphologic and histologic features (Figures 1C-1D). 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 IL-6\textsuperscript{Δ/Δ} mice or WT mice were SP-C-positive and CCSP-negative (Figure 1C). These data indicated that IL-6 suppresses lung tumor initiation induced by K-Ras.

Increased lung tumorigenesis in IL-6\textsuperscript{Δ/Δ} mice is associated with exacerbated lung damage as well as increased number and toxicity of lung macrophages

Before K-Ras\textsuperscript{G12D} induction, the lungs of IL-6\textsuperscript{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-Ras\textsuperscript{G12D} expression in the lungs of WT mice induced mild alveolar congestion and minor impairments of alveolar epithelial integrity, indicating mild lung damage (Figure 2A, left panel, black arrow heads). However, the same K-Ras\textsuperscript{G12D} expression caused more significant lung damage in IL-6\textsuperscript{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 IL-6\textsuperscript{Δ/Δ} mice (Figure 2A, right panel, black arrow heads). These data suggested that IL-6 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 IL-6 suppresses K-Ras-induced lung damage, we compared the activation status of STAT3 in the lung epithelial cells of IL-6\textsuperscript{Δ/Δ} mice and WT mice. It has been well established that one of the most important functions of IL-6 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 to those in WT mice, lung epithelial cells in IL-6ΔΔ mice were with much weaker nuclear staining of STAT3, suggesting a decreased STAT3 activation in these lung epithelial cells in IL-6ΔΔ mice (Figure 2A, empty arrows). These data together suggested that IL-6 protects lungs from K-Ras-induced injury through activation of STAT3 intrinsic to lung epithelial cells.

We also examined the effect of IL-6 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 IL-6 is to regulate immune cells. Like lung epithelial cells, macrophages in the lungs of IL-6ΔΔ mice also showed a significantly decreased STAT3 activation (Figure 2A, filled black arrows). Interestingly, however, significantly more lung macrophages were detected in the lung tissues and BALF of IL-6ΔΔ mice (Figures 2A and 2B). Consistently, the monocyte-attractive chemokines CCL3 (also known as macrophage inflammatory protein-1alpha, MIP-1α) and CXCL2 (also called macrophage inflammatory protein-2alpha, MIP-2α) were significantly increased in the lung tissues of IL-6ΔΔ mice (Figure 2C). Another monocyte-attractive chemokine CXCL1 was also increased, although not statistically significant, in the lung tissues of IL-6ΔΔ 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 IL-6ΔΔ mice and WT mice (Figure S1).

Notably, in comparison to lung macrophages in WT mice, lung macrophages in IL-6ΔΔ mice expressed a higher level of nitric oxide synthase (iNOS), a potent inducer of cell damage (Figure 2D). In line with in vivo data, addition of IL-6 prevented iNOS induction in macrophages in vitro (Figure S2). These data suggested that IL-6 suppresses iNOS expression in lung macrophages. In contrast, lung macrophages in IL-6ΔΔ mice almost completely lost the ability to express IL-10, although
macrophages are the primary source of this anti-inflammatory cytokine (Figure 2D). Nevertheless, this data is highly consistent with the finding that lung macrophages in IL-6Δ/Δ mice are defective in STAT3 activation and with the fact that IL-10 is a transcriptional target of STAT3. Interestingly, IL-6 and IL-10 induced each other in both macrophages and lung epithelial cells (Figure S3, A and B), suggesting a paracrine loop of IL-6/IL-10. More importantly, IL-6 and IL-10 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 (Figure S3, C-E). Given the abundant expression of IL-10 receptor (IL-10R) in lung epithelial cells, these data together suggested that IL-6 and IL-10 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 IL-6Δ/Δ 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 IL-10 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 IL-10-mediated activation and expansion of cytotoxic CD8 T cells is another mechanism by which IL-6 suppresses lung tumorigenesis induced by K-Ras. To test the hypothesis, we first examined the total numbers of T cells in the BALF of IL-6Δ/Δ mice and WT mice. We found that compared to WT mice, IL-6Δ/Δ mice had significantly fewer T cells in their BALF (Figure 3A). The decrease of T cells in the lungs of IL-6Δ/Δ 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 IL-6Δ/Δ mice (Figure 3A). Notably, CD8 T cells in the lungs of IL-6Δ/Δ mice had defective tumor-killing ability. Compared to lung CD8 T cells in WT mice, lung CD8 T cells in IL-6Δ/Δ mice expressed much lower levels of anti-tumor cytokine interferon-gamma (IFN-γ) and apoptosis inducers granzyme A and granzyme B (Figure 3B). Accordingly, lung tumor
cells in IL-6Δ/Δ mice had much lower apoptosis rate (Figure 3C). It seems that the defects of the lung CD8 T cells in IL-6Δ/Δ mice were largely due to their defect in STAT3 activation (Figure 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 IL-6Δ/Δ mice and WT mice. As expected, co-culture with CD8 T cells from WT mice led to loss of lung tumor cells (Figure S4 A). However, CD8 T cells from IL-6Δ/Δ mice significantly lost the tumor killing ability. On the other hand, addition of IL-6 significantly enhanced the tumor killing ability of CD8 T cells (Figure S4 B). Consistently, we found that addition of IL-6 increased expression of cytotoxic molecules in CD8 cells, such as perforin, granzymes A and B, TNFα, and TRAIL (Figure S4, C). Moreover, IL-6 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 (Figure S4, D and E). These data altogether clearly indicated that IL-6 also protects and activates cytotoxic CD8 T cells to suppress K-Ras induced lung tumorigenesis.

IL-6 contributes to the growth of lung cancers induced by K-Ras

Although IL-6Δ/Δ mice developed more lung tumors than WT mice after K-RasG12D induction in lungs, the average sizes of tumors in IL-6Δ/Δ mice were significantly smaller (Figure 4A). This data suggested that IL-6 suppresses the initiation but paradoxically contributes to the growth of lung tumor induced by K-Ras.

To investigate the mechanisms by which IL-6 contributes to lung cancer growth, we first compared the proliferation rates of lung tumors in IL-6Δ/Δ mice and WT mice using the BrdU cell proliferation assay. As shown in Figure 4B, much fewer BrdU-positive cells were detected in the tumors from IL-6Δ/Δ mice compared to those from WT mice, indicating that the tumors in IL-6Δ/Δ mice have a lower proliferation rate. We then compared the STAT3 activation status in these lung tumors in IL-6Δ/Δ mice and WT mice. We found that STAT3 activation was significantly lower in the lung
tumors from IL-6\(^{\Delta/\Delta}\) mice (Figure 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 lung tumors from IL-6\(^{\Delta/\Delta}\) mice (Figure 4D). These data suggested that IL-6 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, IL-6 induced STAT3 activation and cyclin D1 expression, and promoted growth of lung tumor cells \textit{in vitro} (Figure 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 IL-6/STAT3 signaling plays opposite roles in the initiation and growth of lung cancer.

**Discussion**

IL-6 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 IL-6 in cancer cell growth and the association of high IL-6 expression with cancer progression and poor survival of cancer patients (4). In addition, the enthusiasm also comes from clinical trial studies showing that IL-6-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 IL-6 in lung cancer, preventing tumor induction while enhancing tumor growth (Figures 1 and 4). In line with our findings, recent studies indicate that high IL-6 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 IL-6 can only delay lung cancer growth. Thus, to target IL-6 for lung cancer therapy, we need, on one hand, to consider the potential risk in increasing lung damage and tumorigenesis due to long-term IL-6...
inhibition, and on the other hand, to combine IL-6 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 IL-6-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 IL-6-targeted therapy, it could be speculated that the overall clinical benefits of IL-6 therapy alone might be limited, giving both the tumor-suppressing and -promoting roles of IL-6 in lung cancer.

Interestingly, both lung tumor-suppressing and -promoting functions of IL-6 involve its ability in activating STAT3. IL-6 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, IL-6 activates STAT3 in other cells in lungs, particularly macrophages, to express IL-10, which serves as a paracrine stimulus to further enhance lung epithelial STAT3 activation for lung homeostasis under oncogenic stress (Figures 2 and S3). IL-6 also suppresses lung macrophages to express iNOS and thereby prevents iNOS-induced lung damage (Figures 2 and S2). IL-10 produced by lung macrophages, perhaps together with IL-6, also induces STAT3 activation in cytotoxic CD8 T cells for their survival and activation, which in turn induce tumor cell apoptosis (Figures 3 and S4). Thus, it seems that IL-6 utilizes multiple related mechanisms to suppress lung cancer initiation. Paradoxically, IL-6 contributes to lung cancer growth also through STAT3-dependent mechanism. In this case, IL-6 activates STAT3 to induce Cyclin D1 in lung cancer cells, therefore promoting cancer proliferation (Figures 4 and S5). In further support of these findings, our recent studies indicate that mice selectively deficient in lung epithelial STAT3 show overall similar phenotypes as IL-6 deficient mice in K-Ras-induced lung damage, tumor initiation and progression (16).

In summary, these data demonstrate that the IL-6/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 contributing 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 IL-6/STAT3 signaling and the pathogenesis of lung and other cancers associated with IL-6/STAT3 signaling, but also provide a mechanistic basis for targeting IL-6/STAT3 signaling to treat IL-6- and STAT3-associated cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: Z. Qu, G. Xiao

Development of methodology: Z. Qu, F. Sun, J. Zhou

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Z. Qu, F. Sun, J. Zhou, L. Li, L. Xiao, S.D. Shapiro, G. Xiao

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Z. Qu, F. Sun, J. Zhou, L. Li, G. Xiao

Writing, review, and/or revision of the manuscript: Z. Qu, G. Xiao

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Z. Qu, F. Sun, G. Xiao

Study supervision: Z. Qu, G. Xiao

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Acknowledgments

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References


Figure legend

Figure 1. Increased lung tumorigenesis in IL-6Δ/Δ mice after K-RasG12D induction in lungs. A, lung tissues from IL-6Δ/Δ mice and WT mice. Representative tumors are indicated by arrows. B, increased lung tumor multiplicities in IL-6Δ/Δ mice. Data are the mean ± SD (n ≥ 9; *, p < 0.05). C, histological analysis showing increased adenomatous hyperplasia and tumor lesions in the lungs of IL-6Δ/Δ mice. Representative lesions are indicated by arrows. Data are the mean ± SD (n ≥ 9; **, p < 0.01). Scale bar: 200 μm. D. IHC analysis of IL-6, SP-C and CCSP in lung tumors from IL-6Δ/Δ mice and WT mice. Scale bar: 50 μm.

Figure 2. Elevated lung damage and increased lung macrophages in IL-6Δ/Δ mice after K-RasG12D induction in lungs. A, histological and morphological analysis showing increased death rate of lung epithelial cells and decreased STAT3 activation in the lung epithelial cells and lung macrophages in IL-6Δ/Δ mice. Lung epithelial cells and infiltrated macrophages are indicated by empty arrows and filled black arrows, respectively. Damaged lung epithelial cells are indicated by arrowheads. Scale bar: 50 μm. B, hema 3 staining showing more macrophages in the BALF from IL-6Δ/Δ mice. Data are the mean ± SD (n ≥ 5; *, p < 0.05). C, real-time PCR assays showing increased CCL3, CXCL2 and CXCL1 in the lung tissues of IL-6Δ/Δ mice. Data are the mean ± SD (n ≥ 5; *, p < 0.05). D, real-time PCR assays showing increased iNOS but decreased IL-10 in the lung macrophages of IL-6Δ/Δ mice. Data are the mean ± SD (n ≥ 5; *, p < 0.05).

Figure 3. Decreased lung CD8 T cells and tumor cell apoptosis in IL-6Δ/Δ mice expressing K-RasG12D in their lungs. A, hema 3 staining and real-time PCR assays showing decreased CD8 T cells but no
significant change in CD4 T cells in the BALF from IL-6ΔΔ mice. Data are the mean ± SD (n ≥ 5; *, p < 0.05). B, real-time PCR assays showing decreased expression of IFN-γ, granzyme A and granzyme B in the lung CD8 T cells of IL-6ΔΔ mice. Data are the mean ± SD (n ≥ 5; *, p < 0.05). C, IHC assays showing decreased apoptosis of lung tumor cells in IL-6ΔΔ mice. Apoptotic tumor cells were indicated by arrows. D, IF assays showing the lack of STAT3 activation in the lung CD8 T cells in IL-6ΔΔ mice.

**Figure 4.** Decreased cell proliferation and Cyclin D1 expression in K-RasG12D-induced lung tumors in IL-6ΔΔ mice. A, decreased average size of lung tumors in IL-6ΔΔ mice. Data are the mean ± SD (n = 9; *, p < 0.05). B, BrdU labeling showing decreased proliferation rate of lung tumors in IL-6ΔΔ mice. Scale bar: 50 μm. BrdU-positive tumor cells were counted and represented as the percentage of total cells. Data are the mean ± SD (n ≥ 5; *, p < 0.05). C, IHC analysis showing decreased STAT3 activation in lung tumors in IL-6ΔΔ mice. Scale bar: 50 μm. Tumor cells with nuclear staining of STAT3 were counted and represented as the percentage of total cells. Data are the mean ± SD (n ≥ 5; **, p < 0.01). D, IHC analysis showing lower expression levels of Cyclin D1 protein in the lung tumors in IL-6ΔΔ mice. Scale bar: 10 μm. Cyclin D1-positive cells were counted and represented as the percentage of total cells. Data are the mean ± SD (n ≥ 5; **, p < 0.01).
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WT  

IL-6Δ/Δ  

B  

Macrophages in BAL (x10^3)  

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