ORIGINAL ARTICLE

Interleukin-17A Promotes Lung Tumor Progression through Neutrophil Attraction to Tumor Sites and Mediating Resistance to PD-1 Blockade

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ABSTRACT

Introduction: Proinflammatory cytokine interleukin-17A (IL-17A) is overexpressed in a subset of patients with lung cancer. We hypothesized that IL-17A promotes a protumorigenic inflammatory phenotype and inhibits antitumor immune responses.

Methods: We generated bitransgenic mice expressing a conditional *IL-17A* allele along with conditional *Kras*^{G12D} and performed immune phenotyping of mouse lungs, a survival analysis, and treatment studies with antibodies either blocking programmed cell death 1 (PD-1) or IL-6 or depleting neutrophils. To support the preclinical findings, we analyzed human gene expression data sets and immune profiled patient lung tumors.

Results: Tumors in IL-17:Kras^{G12D} mice grew more rapidly, resulting in a significantly shorter survival as compared with that of Kras^{G12D} mice. IL-6, granulocyte colony-stimulating factor (G-CSF), milk fat globule-EGF factor 8 protein, and C-X-C motif chemokine ligand 1 were increased in the lungs of IL17:Kras mice. Time course analysis revealed that levels of tumor-associated neutrophils were significantly increased, and lymphocyte recruitment was significantly reduced in IL17:Kras^{G12D} mice as compared

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Drs. Akbay and Koyama equally contributed to this work.

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with in $Kras^{G12D}$ mice. In therapeutic studies PD-1 blockade was not effective in treating IL-17:$Kras^{G12D}$ tumors. In contrast, blocking IL-6 or depleting neutrophils with an anti–Ly-6G antibody in the IL17:$Kras^{G12D}$ tumors resulted in a clinical response associated with T-cell activation. In tumors from patients with lung cancer with *KRAS* mutation we found a correlation between higher levels of IL-17A and colony- stimulating factor 3 and a significant correlation among high neutrophil and lower T-cell numbers.

Conclusions: Here we have shown that an increase in a single cytokine, IL-17A, without additional mutations can promote lung cancer growth by promoting inflammation, which contributes to resistance to PD-1 blockade and sensitizes tumors to cytokine and neutrophil depletion.

Keywords: Cytokines; IL-17; Neutrophils; MDSC; PD-1; Resistance

Introduction

NSCLC is the leading cause of cancer-related deaths worldwide. Targeted therapies are effective in a subset of lung cancers carrying specific mutations in genes such as *EGFR* or genetic fusions between echinoderm microtubule associated protein like 4 gene (*EML4*) and anaplastic lymphoma receptor tyrosine kinase gene (*ALK*), but there remains a need for therapies specific for *KRAS*-mutant tumors, which constitute about 30% of all adenocarcinomas and are currently refractory to targeted therapies.[1,2] Activating mutations in *KRAS* are associated with smoking and resistance to EGFR inhibitors.[3–5] Smoking has been associated not only with initiation of lung cancer by the carcinogens it carries but also with the promotion of tumor development through induction of inflammation by activation of the nuclear factor κB pathway.[6,7] In preclinical models nuclear factor κB was shown to be required for *Kras*-induced lung tumorigenesis.[8]

The presence of cytokines and inflammatory cells in the lung microenvironment plays a crucial role in determining the outcome of the host antitumor response. Cytokines are released in response to cellular stress, injury, or infection and stimulate the restoration of tissue homeostasis to restrict tumor development and progression. However, persistent cytokine secretion in the setting of unresolved inflammation can promote tumor cell growth, inhibit apoptosis, and drive tumor cell invasion and metastasis.[9] Although the exact mechanisms by which inflammation or inflammatory cells regulate lung cancer growth are not clear, increases in certain components such as circulating interleukin-6 (IL-6)[10] or a higher neutrophil-to–T-cell ratio in lung tumors are associated with a poor prognosis in lung cancer.[11,12]

IL-17A is the prototypical member of the IL-17 family of proinflammatory cytokines. It is produced by T helper 17 cells, CD8 T cells, γδT cells, and natural killer (NK) cells in the tumor microenvironment.[13] Interaction of IL-17 with its receptor, which is expressed on a variety of cell types (including fibroblasts and tumor cells), causes secretion of proinflammatory cytokines such as IL-6, various chemokines, and metalloproteases.[14–18] The inflammatory milieu can contribute to lung cancer growth by further production of tumor-promoting cytokines, reduction in cytotoxic T cells, and development of myeloid-derived suppressor cells.[19] IL-17A and its receptors are expressed across different tumor types; however, their exact role in tumor development, progression, and response to therapeutic regimens is unclear. In melanoma, IL-17A serves as a tumor suppressor; *IL-17A* knockout mice are more susceptible to spontaneous melanoma development.[20] In contrast, *IL-17A* knockout mice are protected from intestinal tumorigenesis in an $AdenoApc^{flox/positive}$ model.[16] Increased presence of IL-17A–positive cells is associated with poor survival in NSCLC.[21,22] IL-17A was shown to be critical in *Kras*-induced lung tumorigenesis in a mouse model lacking *IL-17A* that expressed Kras from the Clara cell promoter, although tumors develop very rapidly in this mouse model.[23]

Although targeted therapies developed against mutant EGFR or echinoderm microtubule associated protein like 4–anaplastic lymphoma kinase proteins are effective only in specific subsets of patients with NSCLC,[24,25] immune checkpoint blockade treatments that activate host antitumor immunity are effective in about 20% of patients with NSCLC across a variety of genotypes.[26] Although tumor or myeloid cell programmed death ligand 1 (PD-L1) expression or increased tumor mutational burden, which can potentially be detected by the immune cells as neoantigens, are associated with a better response to treatment with programmed cell death 1 (PD-1) blockade,[27–29] other predictive biomarkers for response and resistance remain to be discovered. Whether the efficacy of checkpoint blockade is determined by cytokines or the immune cell context of tumors also remains unclear.

IL-17A is expressed at high levels in a subset of lung cancers.[21] Interestingly, we observed that IL-17A could not be detected in bronchoalveolar lavage fluid (BALF) from the previously described $EGFR^{T790M\ L858R}$, $EGFR^{Del19}$, $Kras^{G12D}$, $Kras^{G12D}$:*Tp53* mouse lung cancer models.[30–32] Of note, *Kras*-mutant tumors in this study

were induced after adenovirus administration, which results in fewer lung lesions than in mice in which tumors have been induced by CC10-Cre.[33,34] To characterize the role of IL-17A in *Kras*-mutant lung tumors, we developed a mouse model of chronic inflammation that more closely resembles human *KRAS*-mutant lung cancer by expressing *IL-17A* constitutively in the lung epithelium and then introducing this allele into Lox-Stop-Lox $Kras^{G12D}$-mutant mice. We found that the production of this single cytokine dramatically changed the immune cell dynamics in the tumor microenvironment and promoted resistance to PD-1 blockade.

Materials and Methods

Generation of the IL17 Transgenic Mice

The targeting vector was modified from the original pgkATGfrt vector described.[35,36] The tetracycline-inducible promoter used in the original manuscript was replaced with CAG promoter and Lox-Stop-Lox cassette, which allowed the temporal and spatial control of gene expression. Human IL-17A complementary DNA plasmid (pCR2-IL17) was purchased from Open Biosystems (Waltham, MA). IL-17A complementary DNA was cloned into the targeting vector. Until the stop cassette is removed by Cre recombinase, transgene is not expressed. After cloning the targeting vector and Flippase-expressing plasmid were coelectroporated into the v6.5 C57BL/6(F) × 129/sv(M) embryonic stem (ES) cells (Open Biosystems) with plasmid expressing flippase recombinase.[36] These ES cells are engineered to allow single-copy transgene insertion at the *ColA1* locus. ES clones that carry the IL-17A transgene were selected, expanded, and used to inject into C57BL/6 blastocysts, which gave rise to chimeras. The chimeras were bred with wild-type mice from a BALB/cAnNCrl background (Charles Rive Labs, Boston, MA) to test germline transmission of the transgene. Transgene-positive mice were bred with Kras mice (B6.129S4-Krastm4Tyj/J) obtained from Jackson Laboratories [Bar Harbor, ME]) and expanded for experiments. The mice were maintained in a mixed background strain (C57BL/6, BALB/cAnNCrl, and 129/sv) and given adenovirus-carrying Cre recombinase (titer 5 × 10^6 titer) (University of Iowa) at 6 weeks of age to induce tumors. All mouse experiments were performed with the animal protocol approved by the Dana-Farber Cancer Institute (DFCI) Institutional Animal Care and Use Committee.

Mouse Treatment Studies

Mice were treated with IL-6 antibody (clone MP5-20F3 [BioXcel, Branford, CT]), Ly-6G antibody (clone 1A8 [BioXcel]), and PD-1 antibody (clone 1A12)[37] diluted in saline, 200 μg per dose three times a week by intraperitoneal injection. Magnetic resonance imaging (MRI) was performed with a Bruker Biospec 7 Tesla MRI machine (Bruker, Billerica, MA) at Lurie Family Imaging Center at DFCI.

Cell Line Studies

Mouse cell lines were established from lung tumor nodules from *Kras p53* mice. Tumor tissue was minced and trypsinized, and cells were grown in Roswell Park Memorial Institute 1640 medium containing 10% fetal bovine serum.[38] Cells were sorted for the epithelial marker epithelial cell adhesion molecule to obtain a pure tumor cell population as previously described.[39] Human A549 and H1792 cell lines were purchased from American Type Culture Collection (Manassas, VA). Recombinant human and mouse IL-17 and antimouse IL-17RA antibodies were purchased from PeproTech (Rocky Hill, NJ) and R&D Systems (Minneapolis, MN) (MAB4481), respectively, and used according to the manufacturer's instructions.

BALF Collection and ELISA

BALF was collected by intratracheal injection of 1 mL of saline into the mice after euthanasia and aspiration. The BALF was centrifuged, the cells and debris pelleted, and the supernatant frozen for subsequent analysis. Cytokine and chemokines were measured with enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's protocols. Kits for IL-6 and granulocyte colony stimulating factor (G-CSF) were purchased from (BD Biosciences, San Jose, CA), and milk fat globule-EGF factor 8 protein and C-X-C motif chemokine ligand 1 (CXCL1) were purchased from R&D systems.

Immune Analysis for Mouse Samples

The processing of lung tissue, staining, and flow cytometry analysis were performed as previously described.[40] Mice were euthanatized according to the DFCI Institutional Animal Care and Use Committee–approved animal protocol, and lungs were perfused with cold 5 mM ethylenediaminetetraacetic acid after collection of the BALF. Lung lobes were shredded and incubated in dissociation buffer (100 U/mL of collagenase type IV) (Invitrogen, Carlsbad, CA), 10 μg/mL of Roche DNase I (Sigma-Aldrich, St. Louis, MO), and 10% fetal bovine serum in Roswell Park Memorial Institute 1640 medium for 45 minutes. After dissociation, red blood cells were lysed with red blood cell lysis buffer (Gibco-10492 [Thermo Fisher Scientific, Waltham, MA]), and cell suspensions were passed through a 70-μm cell strainer to isolate single cells. The cell pellet was dissolved in 2% fetal calf serum in Hank's balanced salt

solution and stained with live/dead stain (Life Technologies, Grand Island, NY) per the manufacturer's instructions. Staining antibodies were used at a 1:50 dilution for flow cytometry analysis. For intracellular cytokine staining, cells from whole lungs were fractionated over Ficoll-Paque (GE Healthcare, Pittsburgh, PA) per the manufacturer's instructions. Isolated mononuclear cells were stimulated with 50 ng/mL phorbol myristate acetate (Sigma) and 500 ng/mL Ionomycin (Sigma) for 4 hours in the presence of Golgi plug (BD Biosciences). Fixation/permeabilization buffers (eBioscience, San Diego, CA) or BD Cytofix/Cytoperm buffers (BD Biosciences) were used for intracellular staining. Flow data acquisition was performed on a BD Canto II flow cytometer (BD Biosciences).

Gene Expression Analysis from the TCGA Data Set

In all, 21 *EGFR*- and 74 *KRAS*-mutant samples from The Cancer Genome Atlas (TCGA) data set were used for analysis.[1] Log2 of the fragments per kilobase of transcript per million for CSF-3 and IL-6, and log2 of fragments per kilobase of transcript per million plus 1 for IL-17A from the RNA sequencing data were used for comparisons.

IHC Staining and Quantification

Tissues were fixed overnight in 10% buffered formalin and embedded in paraffin. Ki67 IHC was performed with staining kits for Ki67 (Vector Laboratories, Burlingame, CA) following the manufacturer's protocol. IL-17 immunohistochemistry (IHC) was performed by using Santa Cruz antibody (Santa Cruz Biotechnology, Dallas, TX) at a 1:50 dilution.

For Ki67 staining, Ki67 foci from the images taken at $\times 20$ magnification were quantified; three images per mouse were used.

Cytokine Analysis of Patient Serum Samples

Serum samples were obtained from the Harvard/Massachusetts General Hospital Lung Cancer Study, which is a hospital-based ongoing case-control study initiated in 1992. All cases were histologically confirmed in patients with lung cancer from the Massachusetts General Hospital Cancer Center.[41] Controls were recruited from two sources: (1) case-related controls, who were friends and spouses of lung cancer cases with no specific matching characteristics (all blood-related friends and spouses were excluded) and (2) case-unrelated controls who were friends and spouses of *other* hospital patients (primarily from the oncology and thoracic surgery units out of convenience) for cases without available controls.

Immune Cell Analysis of Freshly Resected Patient NSCLCs

De-identified patient tumors were obtained under institutional review board–approved DFCI protocols 02-180 and 11-104 from subjects providing informed consent for tissue collection. Biopsy specimens were obtained during routine clinical procedures. Human tissue processing and staining were performed following the same procedures as with mouse tissues. All antibodies were used as recommended by the manufacturer.[42]

Results

IL-17A Promotes Lung Tumor Growth by Inducing Production of IL-6

IL-17A is expressed in human lung tumors[21]; however, IL-17A expression in diverse immunocompetent mouse models of lung cancer had not previously been assessed. We analyzed the IL-17A levels in the BALF from a panel of lung genetically engineered murine cancer models with comparable tumor burden. Interestingly, we did not detect IL-17A in the BALF from adenovirus-induced *Kras*-driven (*Kras*^{G12D}, *Kras*^{G12D}: *Tp53)* or doxycycline-inducible *EGFR*-driven (*EGFR*$^{T790M\ L858R\ or}$ *EGFR* ^{del19})[30–32] lung tumors by ELISA (data not shown). To study the potential role of IL-17A in lung cancer pathogenesis mechanistically, we generated a conditional *IL-17A* allele, hereinafter referred to as *IL-17*, that contains the coding region of human *IL-17A* after the floxed stop codon. The stop cassette was removed by adenovirus carrying Cre recombinase[43] to induce IL-17 expression. We crossed IL-17 mice with the conditional Kras^{G12D}-mutant mice and induced the expression of both IL-17 and *Kras*^{G12D} (IL-17:Kras) in mice at 5 to 6 weeks of age by intranasal adenovirus-carrying Cre recombinase delivery (Fig. 1*A* and Supplementary Fig. 1). We confirmed IL-17 expression by IHC on formalin-fixed tissue and by ELISA in BALF (Fig. 1*B* and *C*). IL-17:Kras mice had accelerated lung cancer evidenced by increased tumor burden (Fig. 1*B* and *D*). Tumor cells in IL-17:Kras mouse lungs had a significantly higher proliferation index than those in Kras mouse lungs (Fig. 1*E*), indicating that IL-17 promotes tumor cell growth in mice. Supporting the histological findings, longitudinal analysis revealed significantly reduced survival for IL-17:Kras mice compared with Kras mice (median survival 12 versus 23.2 weeks [$p < 0.0001$]) (Fig. 1*F*). To examine the direct effect of human IL-17 on mouse tumor cells, we first confirmed that *Kras*-mutant lung cancer cells expressed the receptor for IL-17A. IL-17 is recognized by the homodimers or heterodimers of IL-17 receptor A(RA) and IL-17RC.[44] Three independent cell lines derived from murine *Kras*-driven lung tumors were found to express IL-17RA (Supplementary Fig. 2*A*) and respond to both

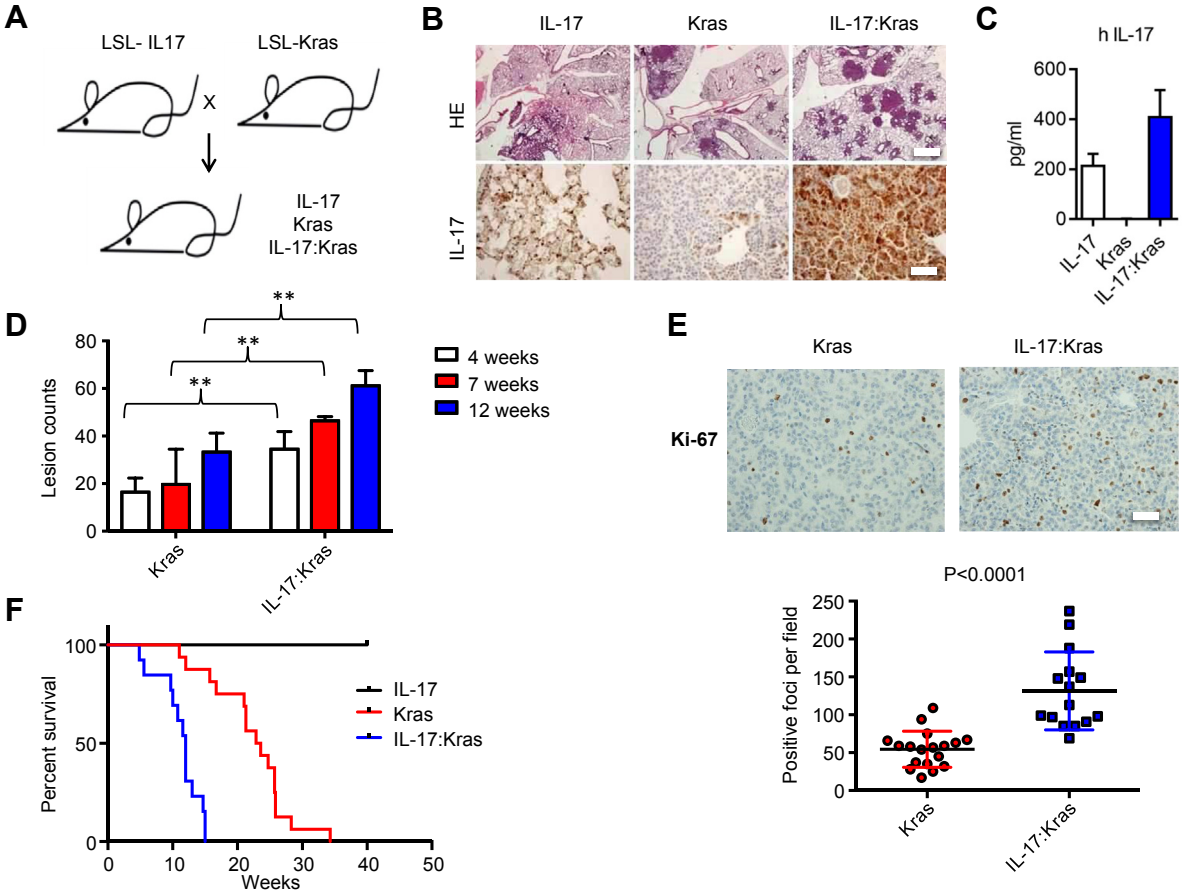

Figure 1. Expression of interleukin-17 (IL-17) in *Kras*-mutant mice promotes lung cancer growth. (*A*) Breeding scheme for the IL-17 and Kras mice. (*B*) Hematoxylin-eosin (HE) staining and IL-17 immunohistochemistry (IHC). Scale bars show 1 mm for the panels depicting HE staining and 50 μm for the panels depicting IL-17 IHC. (*C*) Human IL-17A levels in the bronchoalveolar lavage fluid from the mice detected by enzyme-linked immunosorbent assay: IL-17 (n = 4), Kras (n = 4), and IL-17:Kras (n = 4) mice. (*D*) Total lung lesion counts for mice at 4 weeks (Kras [n = 3] and IL-17:Kras [n = 4] mice), 7 weeks (Kras [n = 4] and IL-17:Kras [n = 4] mice), and 12 weeks (Kras [n = 6] and IL-17:Kras [n = 4] mice) after adenovirus-carrying Cre recombinase induction. (*E*) (*top*) Representative Ki67 IHC and (*bottom*) quantification of Ki67 staining. Scale bars show 50 μm for panels with Ki67 for Kras (n = 6) and IL-17:Kras (n = 5) mice. Three images have been quantified per mouse. (*F*) Kaplan-Meier survival curve for the IL-17, Kras, and IL-17:Kras mice. Mice were considered dead either when they died or when they reached disease burden euthanasia criteria (n = 10, 16, and 13 for IL-17, Kras, and IL-17:Kras mice, respectively). Median survival for Kras mice was 23.2 weeks versus 12 weeks for IL-17:Kras mice ($p < 0.0001$). LSL, Lox-Stop-Sox.

recombinant human and mouse IL-17A by producing IL-6 and granulocyte-colony stimulating factor. This induction was dependent on IL-17RA; when IL-17RA was blocked with antibodies, IL-6 and G-CSF upregulation was impaired (Supplementary Fig. 2*B*). Both IL-6 induction and G-CSF induction by recombinant IL-17A were also confirmed in the human *Kras*-driven lung cancer cell lines A549 and H1792 (Supplementary Fig. 2*C*).

An Increase in IL-17 Level Changes Proinflammatory Cytokine and Cell Profiles in Kras*-Mutant Lung Tumors*

IL-17 is an early proinflammatory cytokine that is responsible for initiating the inflammatory response by inducing the production of several other cytokines in the target cells.[44] To understand how IL-17 affects the cytokine milieu in lungs, we measured the levels of cytokines with an ELISA. Levels of the proinflammatory cytokines IL-6, G-CSF, and milk fat globule-EGF factor 8 protein and the chemokine CXCL1 were significantly increased in the BALF of IL-17:Kras mice as compared with Kras mice with the same disease burden (Supplementary Fig. 3*A* and *B* [12-week time point]). Time course analysis of the cytokines and chemokines revealed that as the tumors progressed, the proinflammatory cytokines were increased, except for CXCL1, which peaked at week 7 (Fig. 2*A*). To test whether the increase in the levels of proinflammatory cytokines and the patterns of cytokine level increase reflected the immune cell infiltration into the tumors, we analyzed the

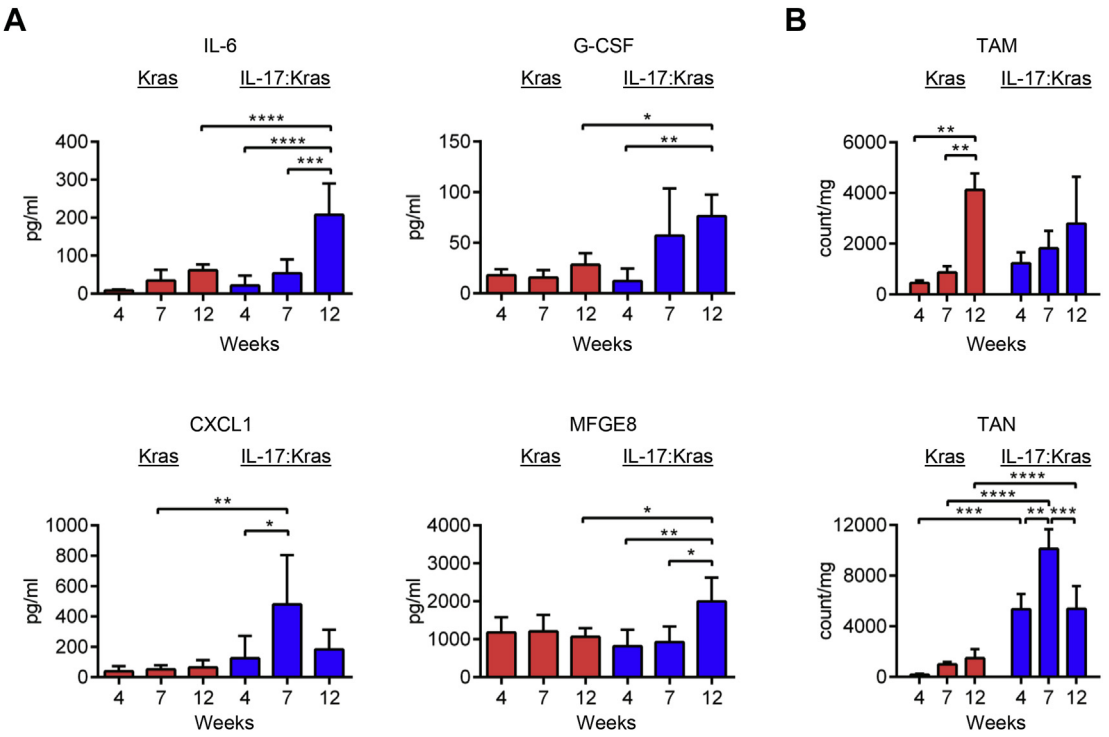

Figure 2. Interleukin-17 (IL-17) modulates the lung cytokine and immune cell profiles. (*A*) Cytokine and chemokine (IL-6, granulocyte colony-stimulating factor [G-CSF], C-X-C motif chemokine ligand 1 [CXCL1], and milk fat globule-EGF factor 8 protein [MFGE8]) analysis of bronchoalveolar lavage fluid at 4, 7, and 12 weeks in Kras and IL-17:Kras mice (n = 4 at 4 weeks, n = 4 at 7 weeks, and n = 5 at 12 weeks for both Kras and IL-17:Kras mice). (*B*) Tumor-associated macrophage (TAM) and tumor-associated neutrophil (TAN) counts per milligram of the lung tissue in Kras and IL-17:Kras mice (n = 4 at 4 weeks, n = 4 at 7 weeks, and n = 5 at 12 weeks for both Kras and IL-17:Kras mice).

dynamics of immune cell types at 4, 7, and 12 weeks after tumor induction. These time points were determined on the basis of survival of double-mutant mice. Correlating with the high levels of IL-6 and G-CSF, the counts of tumor-associated neutrophils (TANs) were increased in IL-17:Kras mice, making TANs the most dominant cell population. TAN counts were highest at 7 weeks after tumor induction (Fig. 2*B*), correlating with CXCL1 production. CXCL1 can be produced by tumor cells and by neutrophils themselves in a positive feedback loop,[45] which may explain the observed pattern.

Neutrophil Recruitment to Tumors Changes T-Cell Counts and Activation

Given the importance of adaptive immunity in suppressing growth of lung tumors, we next analyzed T-cell counts and phenotypes in IL-17–expressing tumors. Analysis of tumor burden and CD8 and CD4 T-cell counts in Kras and IL-17:Kras mice revealed significant increases of CD4 and CD8 T-cell counts with disease progression in *Kras*-mutant mice. In contrast, IL-17:Kras mice did not show such an increase but did show a trend toward decreased T-cell counts with increased disease burden (Fig. 3*A*). IL-17:Kras tumors had significantly fewer CD4 T cells, CD8 T cells, and B cells and more TANs than Kras tumors at the same disease burden (lung weight) (Supplementary Fig. 3*C*). Counts of other cell types showed distinct patterns among Kras and IL-17:Kras mice at different time points. CD103-positive dendritic cell counts were increased significantly between 4 and 12 weeks in Kras mice, whereas IL-17:Kras tumors showed significantly fewer CD103-positive dendritic cells at 12 weeks compared with Kras tumors (Supplementary Fig. 4*A*). Eosinophil counts peaked at 7 weeks for *Kras*-mutant tumors whereas inflammatory monocytes did not show any significant changes (see Supplementary Fig. 4A). We also performed direct correlations of the counts of B and NK cells and disease burden. B-cell counts and tumor burden had a significant positive correlation in Kras mice, whereas IL-17:Kras mice did not show this trend. NK cell counts remained the same for Kras mice, whereas in IL-17:Kras mice they showed a negative correlation with disease burden (Supplementary Fig. 4*B*). These findings suggest that tumors do not have a static immune context and that cell composition is subject to change at different time points that correlate with changes in cytokines and chemokines.

We also analyzed immune checkpoint receptor and ligand expression in the Kras and IL-17:Kras tumors at

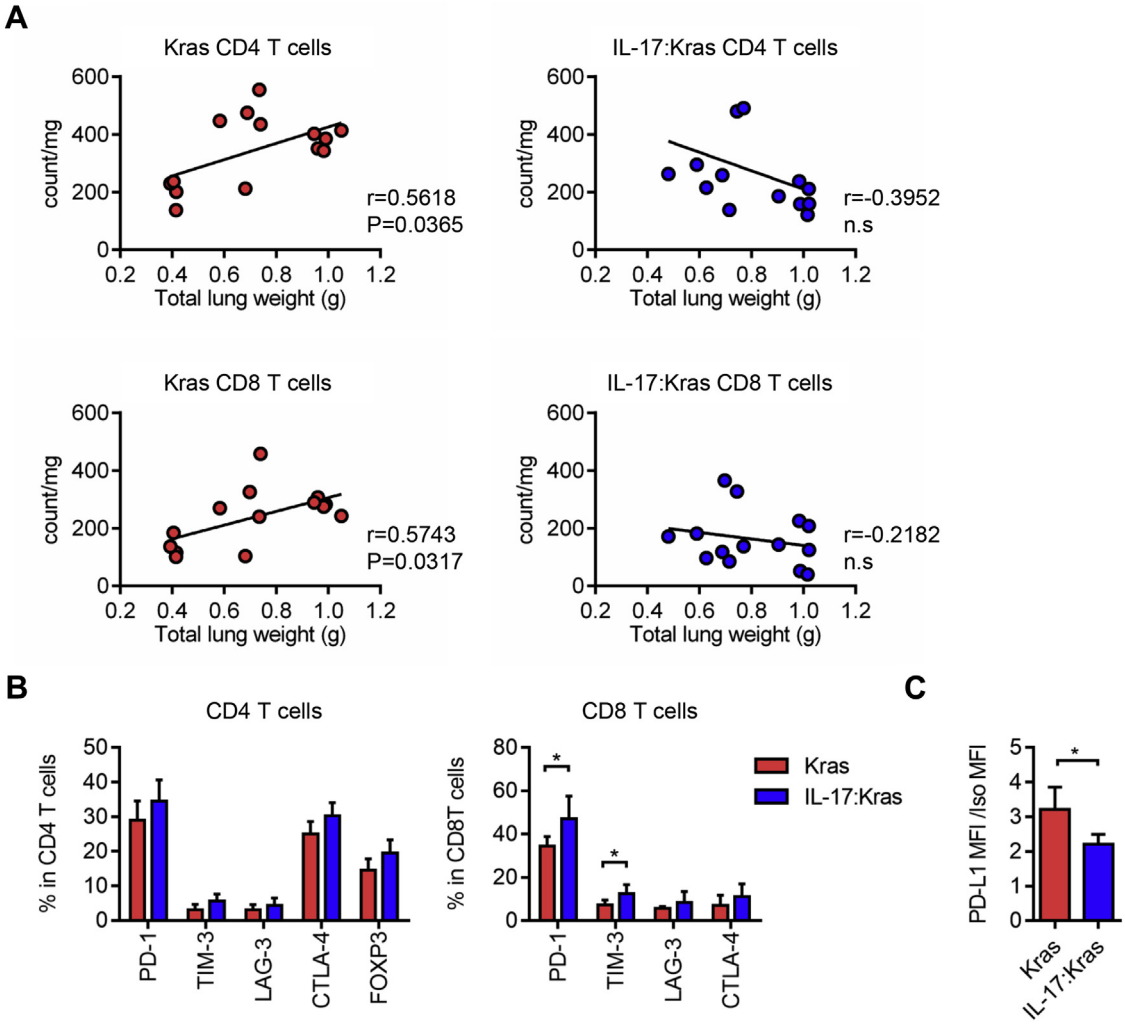

Figure 3. Interleukin-17 (IL-17) modulates T cell infiltration to tumor sites and immune checkpoint receptor expression. (*A*) Correlations of CD4 and CD8 T-cell counts and total lung weights for Kras mice (n = 14) and IL-17:Kras mice (n = 14). (*B*) Immune checkpoint receptor expression in CD4 and CD8 T cells from the Kras versus IL-17:Kras mice at the same tumor burden (see Supplementary Fig. 3*A*, Kras [n = 5] and IL-17:Kras [n = 5] mice). (*C*) Programmed death ligand 1 (PD-L1) expression in the tumor cells from Kras versus IL-17:Kras mice at the same tumor burden (see Supplementary Fig. 3*A*, Kras [n = 5] and IL-17:Kras [n = 5] mice). n.s., not significant; TIM-3, T-cell immunoglobulin and mucin domain-3; LAG3, lymphocyte activating 3; CTLA4, cytotoxic T-lymphocyte associated protein 4; FOXP3, forkhead box P3; MFI, median fluorescence intensity.

the same disease burden (Supplementary Fig. 3*A*). There was a significant increase in PD-1 and T-cell immunoglobulin and mucin domain 3 expression in the CD8 T cells of IL-17:Kras tumors at the 12-week time point (Fig. 3*B*) as compared with in those of Kras tumors. At this time point, tumor cell PD-L1 expression was significantly lower in IL-17:Kras tumors compared with in Kras tumors (Fig. 3*C*).

IL-17:Kras Tumors Are Resistant to Immune Checkpoint Blockade

Given that IL-6 is a tumor-promoting cytokine and is involved in both recruiting tumor-promoting neutrophils[46] and driving tumor growth directly,[47] we explored whether blockade of IL-6 or reducing neutrophil recruitment could inhibit tumor growth in our models. We treated IL-17:Kras mice with an IL-6–blocking antibody or a neutrophil-depleting Ly-6G antibody. IL-6– and Ly-6G antibodies decreased the tumor burden significantly, as confirmed by MRI, at 2 weeks after treatment (Fig. 4*A* and *B*) ($p = 0.0086$ and $p = 0.0107$ for untreated versus IL-6 antibody–treated and untreated versus Ly-6G antibody–treated, respectively). In contrast, tumors in IL-17:Kras mice did not respond to PD-1–blocking antibody (1A12)[37] (Fig. 4*A* and Supplementary Fig. 5*A*). In longitudinal comparative studies, IL-17:Kras mice did significantly worse than Kras mice in response to PD-1 antibody

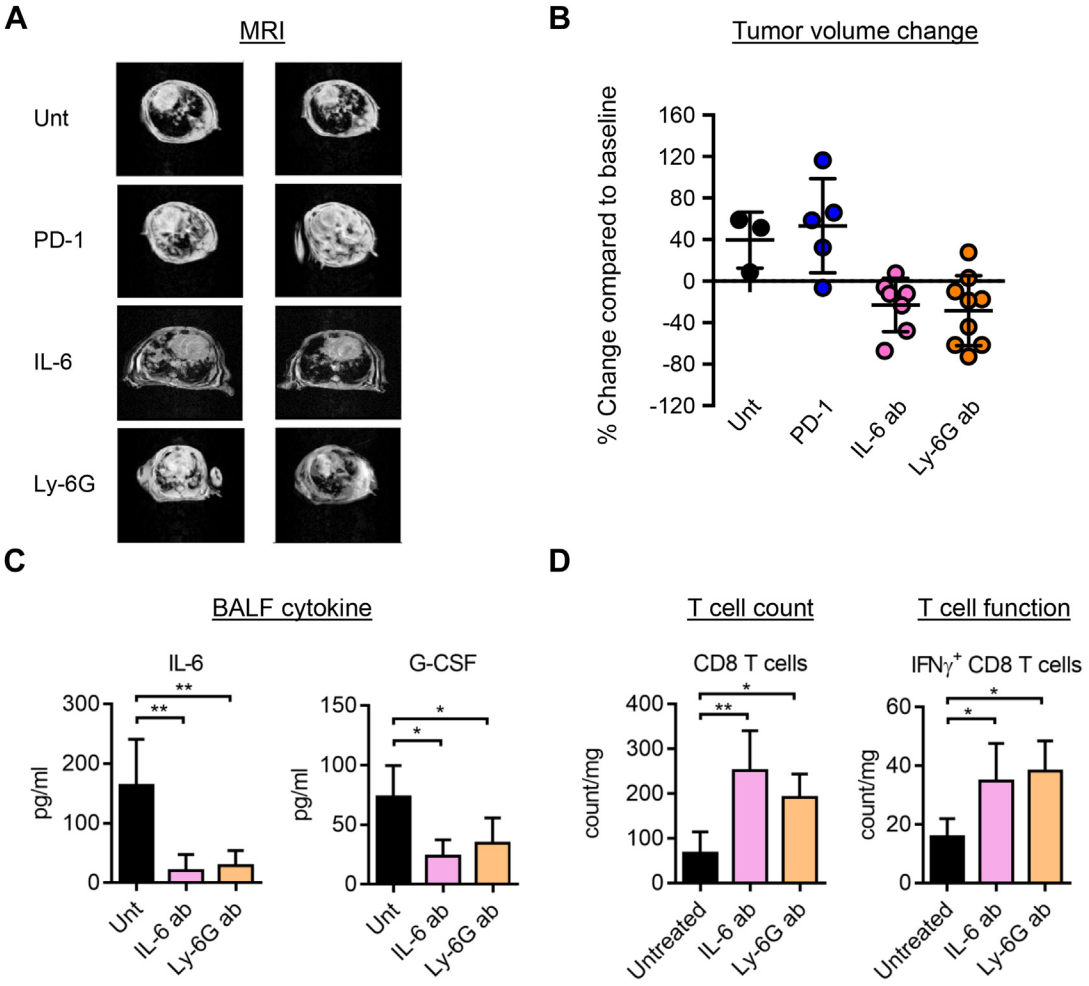

Figure 4. High-interleukin-17 (IL-17) tumors are resistant to programmed cell death 1 (PD-1) immune checkpoint blockade but sensitive to IL-6 blockade or neutrophil depletion. (*A*) Representative magnetic resonance imaging (MRI) images (*left*) and quantification of MRI images (right) of mice untreated (Unt) or treated with PD-1-, IL-6-, or Ly-6G-blocking antibodies (abs) for 2 weeks. (*B*) Quantification of MRI for treatments in (*A*). Each data point represents a different mouse. (*C*) Levels of IL-6 and granulocyte colony-stimulating factor (G-CSF) in the bronchoalveolar lavage fluid (BALF) of IL-17:Kras mice Unt (n=4) or treated with IL-6 (n = 5) or Ly-6G (n = 5) abs for 2 weeks. (*D*) CD8 T-cell counts (*left*) and intracellular interferon gamma (IFNγ)-positive CD8 T-cell counts (*right*) per milligram of lung for IL-17:Kras mice either Unt (untreated) or treated with IL-6 or Ly-6G abs. Unt (n = 4), treated with IL-6 ab (n = 5), and Ly-6G ab (n =5).

(Supplementary Fig. 5) (mean response +24% versus +68% for Kras and IL-17:Kras, respectively [n = 10 for both groups, $p = 0.0005$]).

To determine the mechanism of response to IL-6– or Ly-6G–blocking antibodies, we analyzed the cytokines and T cells in mice treated with IL-6 or Ly-6G antibodies. Free IL-6 and G-CSF levels were reduced in the BALF of treated mice (Fig. 4*C*). There was also a significant increase in the total CD8 T-cell counts, and an increase in CD8 T cells expressing interferon gamma, a cytokine associated with T-cell function (Fig. 4*D*). Blocking IL-6 also led to a significant decrease in proliferation of the tumor cells (Supplementary Fig. 5*B* and *C*). These data suggest that expression of IL-17 is sufficient to change the immune microenvironment and that this change is dependent at least in part on IL-6 and immune suppressive neutrophils. Blocking either of these inhibitory pathways causes an enhanced anti-tumor immune response with increased T-cell activation in the microenvironment, and suppression of tumor growth.

Kras-*Mutant Lung Tumors Express IL-17A, and Neutrophil Counts Are Negatively Correlated with T-Cell Counts in Patient NSCLC Samples*

To validate our preclinical findings, we first analyzed serum samples from patients with lung cancer. Interestingly, serum samples from patients with lung cancer had higher levels of IL-17A as compared with those of

healthy controls (Fig. 5*A*). Next, we studied the proinflammatory cytokines and myeloid cells in the context of NSCLCs of the adenocarcinoma histological type. In samples from the TCGA database,[1] *KRAS*-mutant tumors, which are associated with smoking and inflammation, displayed significantly higher levels of IL-17A and G-CSF as compared with *EGFR*-mutant tumors (Fig. 5*B*) ($p = 0.049$ and 0.001, respectively). IL-6 levels were comparable in *EGFR*- and *KRAS*-mutant tumors, likely owing to the fact that IL-6 can be produced by several types of cells in the lung microenvironment.[39] To further explore the relationship among neutrophils and T cells in human lung cancers, and the hypothesis that neutrophils limit antitumor T-cell recruitment to tumor sites, we analyzed surgically resected NSCLC samples. As observed in the mouse studies, surgically resected patient lung tumors showed a significant negative correlation between the counts of TANs (CD45 positive, CD66b positive) and T cells (CD45 positive, CD3 positive), shown as the percentage of cells in total CD45-positive cells ($r = -0.7767$, $p = 0.0001$, calculated by linear regression analysis) (Fig. 5*C*). Given the importance of T-cell recruitment in lung tumor prognosis and response to checkpoint blockade treatments, identifying patients with high levels of neutrophil infiltration will be important to develop strategies to overcome barriers to antitumor T-cell activity in the context of an immune microenvironment that is unlikely to be responsive to PD-1 blockade alone.

Discussion

Our studies suggest that IL-17 is protumorigenic by inducing production of IL-6 and recruitment of

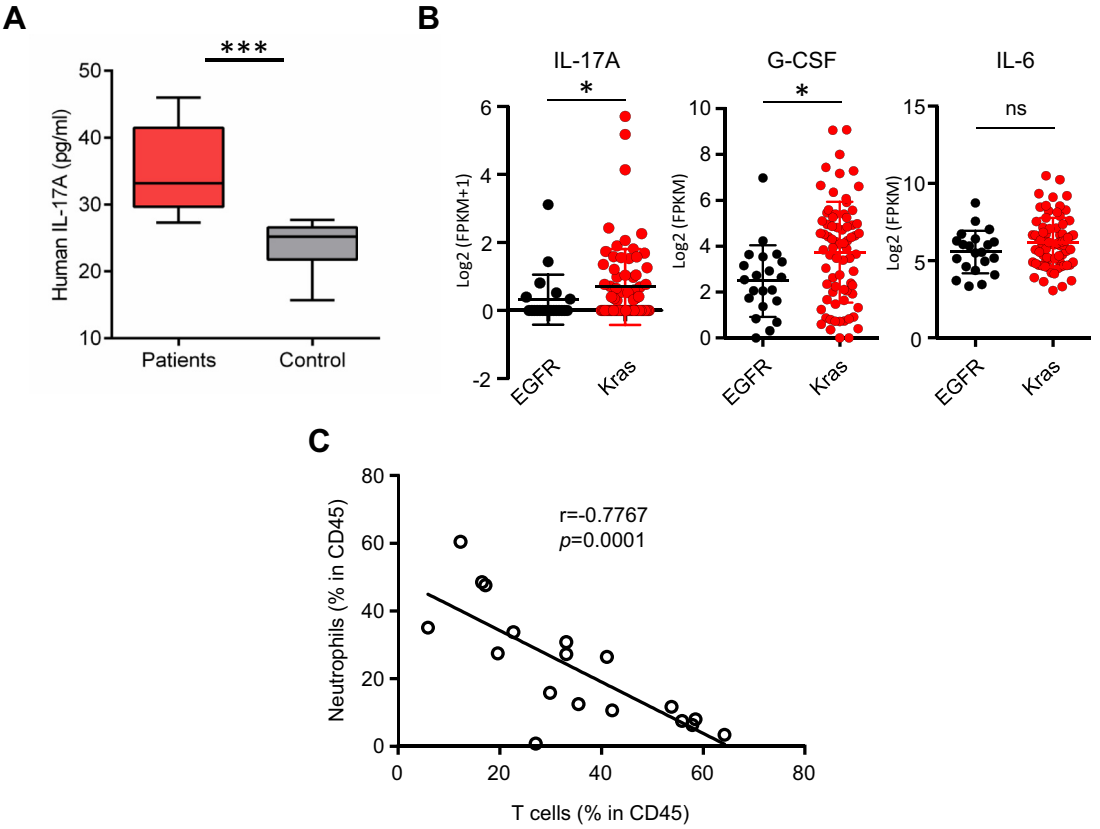

Figure 5. Patients with lung cancer have increased serum interleukin-17 (IL-17) levels. The increase in neutrophils is associated with a decrease in lymphocytes. (*A*) IL-17A levels in the sera of patients with lung cancer (n = 10) and healthy controls (n = 9) ($p = 0.0003$). (*B*) Expression of IL-17A, granulocyte colony-stimulating factor (G-CSF) (colony-stimulating factor 3), and IL-6 in EGFR and *Kras*-mutant lung cancer samples from The Cancer Genome Atlas data set EGFR (n = 21) and Kras (n = 75) samples.[1] Expression of IL-17A, colony-stimulating factor 3, and IL-6 were calculated from log2 values of the RNA sequencing data ($p = 0.049$, $p = 0.0103$, and $p = 0.1546$ for IL-17A, G-CSF, and IL-6, respectively). *p* Values were calculated by using the Mann-Whitney test in GraphPad Prism software (GraphPad Software, La Jolla, CA). (*C*) Correlations of neutrophils (CD45 positive, CD66b positive) and T cells (CD45 positive, CD3 positive) in freshly resected samples from patients with NSCLC (Kras-mutant) were calculated as the percentage of immune cells in the total CD45-positive cells in all samples. *p* Value was calculated by linear regression by using GraphPad Prism software (n = 18). ns, not significant; FPKM, fragments per kilobase of transcript per million.

neutrophils in the tumor immune microenvironment. An increase in IL-17A is sufficient to change lung cytokine secretion and associated myeloid cell phenotypes, T-cell function, and disease course. We found that the cytokine profile and myeloid cell counts changed over time, suggesting that tumor immune context is dynamic. Our findings support previous studies that showed a link between high neutrophil-to–T-cell ratio in clinical samples and a poor prognosis of patients.[48] Our studies suggest that TANs support lung cancer development and progression. Previous studies reported increased lung tumor metastasis with IL-17 expression in patients.[49] We have not detected increased metastasis in this model, likely because of a lack of additional tumor suppressor mutations, such as loss of tumor protein p53 gene (*Tp53*).[50]

Our analysis revealed an increase in circulating levels of IL-17A in human lung patients with adenocarcinoma. We previously showed that a high incidence of lung adenocarcinomas similarly develops in mice that are compound deficient in granulocyte-macrophage colony-stimulating factor, IL-3, and interferon gamma against a background of chronic inflammation and infection.[51] The IL-17 tumor-promoting axis was activated in this lung carcinoma model.

We have previously demonstrated that oncogenic *Kras:Lkb1*-driven murine lung tumors are associated with a dense inflammatory infiltrate composed predominantly of neutrophils[39] and are resistant to PD-1 checkpoint blockade. Combined with this current study, these models suggest that the IL-17-neutrophil axis is important in lung cancer inflammation and in shaping the lung cancer immune microenvironment.

Response to PD-1 checkpoint blockade has been shown to be associated with PD-L1 expression, PD-1–positive T-cell counts, or T-cell presence in the tumor tissue.[52] By studying another mouse lung tumor model, *Kras:Lkb1*, we showed that *Lkb1* inactivation modulates the lung immune phenotype and promotes resistance to checkpoint blockade by inducing neutrophilic myeloid-derived suppressor cells.[39] Our current study provides a unique example of oncogene/tumor suppressor mutation-independent phenotype associated with a single cytokine. Increased cytokine levels have been associated with resistance to targeted therapies. High IL-17 level has been associated with resistance to vascular endothelial growth factor inhibitors,[53] and IL-6 has been previously implicated in resistance to EGFR or MEK inhibitors.[54,55] Our findings suggest that IL-17 may also be a key molecule important in suppressing T cells and mediating resistance to PD-1 immune checkpoint blockade.

The primary role of IL-17 is to recruit and activate neutrophils during an infection.[44,56] IL-17 induces the production of proinflammatory cytokines from stromal cells and tumor cells. We have shown that IL-17 induces IL-6 and G-GSF in lung tumor cells in the mouse, but the mechanism by which IL-17 is elevated in patients with lung cancer remains unclear. Here we show that IL-17 contributes to lung tumor pathogenesis by coordinating a network of cellular and soluble factors that facilitate cell transformation and progression while antagonizing protective antitumor lymphocyte cytotoxic function. These investigations will help identify novel host targets such as IL-17 itself or IL-17–induced chemokines or cytokines for cancer therapy, which may complement other efforts to develop lung cancer treatments based on tumor cell–intrinsic defects or activation of adaptive immunity against cancers by checkpoint blockade treatments. Future studies should focus on the mechanisms regulating the levels of cytokines, such as genomic alterations, smoking history, and/or epigenetic modifications,[57] as well as on the impact of these mechanisms in mediating resistance to conventional chemotherapy and immunotherapies.

It remains to be determined whether the changes in the lung microenvironment of smokers also contribute to the increase in IL-17 and involve, at least in part, initiation of an infection response. Recent studies in an inflammatory colon cancer model suggests there are other sources of IL-17, such as $\gamma\delta$T cells of the innate immune system, that promote the chronic inflammatory state critical for infection-driven colon tumor development[58] and metastasis of breast tumors.[17] Previous studies have also shown that nontypeable *Haemophilus influenzae* induces inflammation similar to chronic obstructive pulmonary disease. It has been shown that this type of inflammation induces T helper 1 and T helper 17 cell recruitment in lung tumors in experimental systems.[23] The microbiome of patients with lung cancer may indeed be variable, causing differences in disease outcome and response to treatments, and may also possibly be associated with toxicities seen with immunotherapies.

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Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of the *Journal of Thoracic Oncology* at www.jto.org and at http://dx.doi.org/10.1016/j.jtho.2017.04.017.

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