T helper 17 cells play a critical pathogenic role in lung cancer

Seon Hee Chang¹, Seyedeh Golsar Mirabolfathinejad, Harshadadevi Katta, Amber M. Cumpian, Lei Gong, Mauricio S. Caetano, Seyed Javad Moghaddam, and Chen Dong

Departments of ¹Immunology and ²Pulmonary Medicine, Center for Inflammation and Cancer, The University of Texas MD Anderson Cancer Center, Houston, TX 77034

Edited by Ruslan Medzhitov, Yale University School of Medicine, New Haven, CT, and approved March 11, 2014 (received for review October 10, 2013)

Lung cancer development is associated with extensive pulmonary inflammation. In addition, the linkage between chronic obstructive pulmonary disease (COPD) and lung cancer has been demonstrated in population-based studies. IL-17-producing CD4 helper T cells (Th17 cells) play a critical role in promoting chronic tissue inflammation. Although Th17 cells are found in human COPD and lung cancer, their role is not understood. We have thus used a mouse model of lung cancer, in which an oncogenic form of K-ras (K-rasG12D), frequently found in human lung cancer, is restrictedly expressed in lung epithelial cells [via Clara cell secretory protein (CCSP) CRE]. In this model, Th17 and Th15 but not Th1 cells were found enriched at the tumor tissues. When CCSPCre/K-rasG12D mice were weekly challenged with a lysate of nontypeable Haemophilus influenzae (NTHi), which induces COPD-type inflammation and accelerates lung cancer development, they showed greatly enhanced Th17 cell infiltration in the lung tissues. Lack of IL-17, but not IL-17F, resulted in markedly delayed tumor growth, which was correlated with reduced STAT3 phosphorylation and NFκB activation in tumor cells. In addition, the linkage between chronic obstructive pulmonary disease (COPD) and lung cancer has been demonstrated in population-based studies. IL-17 expression was not only increased in lung adenocarcinoma (13). The density of infiltrating Th17-positive cells in primary human nonsmall cell lung cancer was inversely correlated with patient outcome and correlated with smoking status of the patients (14). Th17 cells specific for a common tumor antigen were found in lung cancer patients as part of their spontaneous immune response to the autologous tumor (15). However, the function of Th17 cells and IL-17 in the development of lung cancer remains to be shown. Animal model studies have revealed contrasting roles of IL-17 in various tumors (16). Tumor-promoting effect of IL-17 was shown in some models such as colon cancer (17–20), whereas in others, IL-17 supported anti-tumor immunity, including in B16 melanoma model (21–24). Thus, the role of IL-17 could be complex and tumor-specific.

To properly evaluate the role of IL-17 in inflammation-associated lung cancer, we used a model of oncogenic K-ras mutation expressed only in the lung. Mice expressing K-ras mutation in Clara cells (CCSPCre/K-rasG12D mice) spontaneously develop lung adenocarcinoma (25). In addition, we induced COPD-type lung inflammation by challenging mice with lysates of nontypeable Haemophilus influenzae (NTHi). Inflammation driven by NTHi can promote tumor growth in CCSPCre/K-rasG12D mice (25). These experiments collectively indicate a tumorigenic role of IL-17–mediated inflammation in the development of lung cancer.

**Significance**

One of the challenging problems in lung cancer is to understand how inflammation pathways influence on the development of lung cancer and to identify early immune mediators. In this study, we functionally dissect the critical events occurring at the interface between endogenously arising lung tumors and the host immune system that determine tumor progression. Our findings greatly advance our knowledge on the function of T helper 17 cells in lung cancer and help understand the mechanisms of inflammatory mediators that promote lung cancer.

**Author contributions:** S.H.C., S.J.M., and C.D. designed research; S.H.C., S.G.M., H.K., A.M.C., L.G., M.S.C., and S.J.M. performed research; S.H.C., M.S.C., and S.J.M. analyzed data; and S.H.C. and C.D. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. Freely available online through the PNAS open access option.

¹To whom correspondence may be addressed. E-mail: shchang@mdanderson.org or smoghadd@mdanderson.org.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1319051111/-/DCSupplemental.
Results

Th17 Cells Preferentially Accumulate in a Model of Lung Cancer. Although Th17 cells are found in human COPD and lung cancers, their functional roles have not been understood. To conclusively address this issue, we adopted a mouse model of lung adenocarcinoma (CCSP\textsuperscript{Cre}/K-ras\textsuperscript{G12D}) where oncogenic form of K-ras (K-ras\textsuperscript{G12D}) (26) is restrictedly expressed in lung epithelial cells (CCSP\textsuperscript{Cre}) (27), hereinafter referred to as CC-LR. CC-LR mice develop lung adenocarcinoma without any potential of metastasis (25). K-ras activation in the bronchiolar epithelium has been associated with a robust inflammatory response (28), therefore providing an ideal spontaneous tumor model to study cancer-associated inflammation regulation. Analysis of bronchoalveolar lavage fluid (BALF) in 14-wk-old CC-LR mice, with visible tumors on lung surface and lung tissue with pulmonary hyperplasia and adenomas, revealed that there is an increased expression of Th17 signature cytokines IL-17, IL-17F, and IL-22 (Fig. 1A). In contrast, IFN\gamma were not increased in this model. To understand the source of these cytokines in CC-LR mice, we took either BALF cells or mononuclear fraction from tumor-bearing lung and restimulated with phorbol myristate acetate (PMA) and ionomycin for intracellular cytokine staining of lung mononuclear cells indicated that over 54% (CC-LR 6.54 ± 0.91%, CC-LR 16.48 ± 2.71, P < 0.01) but similar to untreated CC-LR mice. Percentages of IL-17\gamma\delta T cells, however, remained constant between NTHi-exposed WT and NTHi-exposed CC-LR mice (Fig. 2B and C). These results indicate that NTHi challenge in mice preferentially promotes Th17 cell response and an expansion of Th17 cells is associated with increased tumor growth in the presence of NTHi-induced type of inflammation.

Increased Th17 Cells Associate with Inflammation-Promoted Lung Cancer. NTHi, found in lower airways of patients with COPD, is associated with exacerbation of COPD (29). Interestingly, chronic exposure to NTHi led to COPD-type lung inflammation characterized with an expanded Th17 cell population in the lung tissues of normal mice (Fig. 2A). NTHi challenge in CC-LR mice further accelerated the production of Th17 related cytokines in comparison with NTHi in WT mice (Fig. 2A). Intracellular cytokine staining of lung mononuclear cells indicated that over 25% of CD4 T cells in lung were stained with anti–IL-17, whereas IFN\gamma\gamma Th1 cells among CD4 T cells were decreased upon NTHi challenge (Fig. 2B). Notably, IL-17-deficient mice exhibited not only reduced inflammatory cell infiltration but also a decrease in airway wall thickness after 25 wk of NTHi challenge (Fig. S2).

It has been also shown that NTHi challenge in CC-LR mice resulted in lung cancer promotion (25). We found that in NTHi-exposed CC-LR mice in comparison with untreated CC-LR or NTHi-treated WT mice, percentages of Th17 cells among CD4 population were increased (NTHi WT 31.67 ± 1.45%, NTHi CC-LR 45.25 ± 4.42%, P < 0.05). In NTHi-exposed CC-LR mice, percentage of IFN\gamma\gamma CD4 cells was increased in comparison with NTHi-exposed WT mice (NTHi WT 6.40 ± 0.53%, NTHi CC-LR 16.48 ± 2.71, P < 0.01) but similar to untreated CC-LR mice. Percentages of IL-17\gamma\delta T cells or Foxp3\gamma regulatory CD4 T cells, however, remained constant between NTHi-exposed WT and NTHi-exposed CC-LR mice (Fig. 2B and C). These results indicate that NTHi challenge in mice preferentially promotes Th17 cell response and an expansion of Th17 cells is associated with increased tumor growth in the presence of NTHi-induced type of inflammation.

IL-17 Deficiency Inhibited Lung Cancer Development. The above results suggest a potential role for Th17 cells in lung cancer. To evaluate this possibility directly, CC-LR mice were crossed with Il17\textsuperscript{−/−} mice. Tumor numbers on the surface of mouse lung were counted at the age of 14 wk. Lack of IL-17 resulted in a ∼75% (52.7 ± 7.1 in CC-LR Il17\textsuperscript{+/+} vs. 13.2 ± 2.5 in CC-LR Il17\textsuperscript{−/−}, P = 0.003) reduction in lung surface tumor numbers compared with age- and sex-matched CC-LR Il17\textsuperscript{+/+} mice (Fig. 3A). The mean size of adenomas in CC-LR Il17\textsuperscript{−/−} mice were smaller than that in CC-LR Il17\textsuperscript{+/+} mice (Fig. 3C and Fig. S3). In contrast, tumor numbers in CC-LR Il17\textsuperscript{+/+} mice were similar to those in CC-LR Il17\textsuperscript{−/−} mice (Fig. 3A and C). Together, these results indicate that IL-17, but not IL-17F, is required for lung tumor development.

The majority of recovered cells in BALFs of 14-wk-old tumor-bearing CC-LR mice were macrophages. CC-LR Il17\textsuperscript{+/+} mice showed reduced total white blood cell (WBC) and macrophage numbers in comparison with CC-LR Il17\textsuperscript{−/−} mice (Fig. 3B). The number of infiltrated lymphocytes and neutrophils were also significantly reduced in CC-LR Il17\textsuperscript{−/−} mice in comparison with untreated CC-LR or NTHi-exposed CC-LR mice (Fig. 3B and Table S1). In contrast, we did not observe any difference in the number of infiltrated cells in lung between CC-LR Il17\textsuperscript{+/+} and CC-LR Il17\textsuperscript{−/−} mice (Fig. 3B). To determine whether IL-17 is involved in tumor promotion by NTHi-induced inflammation, CC-LR Il17\textsuperscript{−/−} mice were challenged with NTHi. In comparison with CC-LR Il17\textsuperscript{+/+} mice, the numbers of visible tumors on the lung surface of CC-LR Il17\textsuperscript{−/−} mice were reduced by ∼54% (CC-LR Il17\textsuperscript{+/+} 112 ± 7, CC-LR Il17\textsuperscript{−/−} 52 ± 5, P < 0.001) after weekly NTHi exposure for 4 wk from the age of 10 wk (Fig. 3D). CC-LR Il17\textsuperscript{−/−} mice had more hyperplastic early-stage lesions and smaller tumors in comparison with CC-LR Il17\textsuperscript{+/+} mice. We found the number of neutrophils, macrophages, and lymphocytes in BALF were also reduced in CC-LR Il17\textsuperscript{−/−} mice (Fig. 3E and Table S2). Therefore, our results indicate that IL-17 is critical in inflammation-associated lung adenocarcinoma.

IL-17 Regulates Cancer Cell Proliferation, Angiogenesis, and Production of Proinflammatory Mediators. To address how IL-17 promotes lung cancer, the proliferation index of tumor cells in situ was
and tumor showed an accumulation of Gr-1⁺CD11b⁺ myeloid cells in CC-LR, in comparison with normal lung. During tumorogenesis, a heterogeneous population of myeloid cells, known as myeloid-derived suppressive cells, is generated (5). Myeloid-derived suppressive cells express the cell-surface markers Gr-1 and CD11b and function to suppress T-cell activity. We found a 77% reduction in total number of Gr-1⁺CD11b⁺ cells among CD45⁺ fraction of lung mononuclear cells in IL-17-deficient mice (CC-LR Il17⁻/⁻, 8.78 ± 2.24; CC-LR Il17⁺/⁺, 2.79 ± 1.42; P = 0.027, expressed as x10⁶ cells) (Fig. 4E).

To evaluate the function of Gr-1⁺CD11b⁺ cells, we examined their immune suppressive activity in vitro. Gr-1⁺CD11b⁺ cells were isolated from tumors of CC-LR mice and from lung parenchyma of WT mice. When Gr-1⁺CD11b⁺ cells were cultured with total splenocytes in the presence of αCD3 and αCD28, Gr-1⁺CD11b⁺ cells isolated from tumors exhibited suppressive activity to T-cell proliferation and cytokine production (Fig. S4B). This finding is in agreement with a previous study using CC10 promoter-driven SV40 TAg transgene model of lung adenocarcinoma (30). This result suggests that CC-LR Il17⁻/⁻ may have increased cytotoxic CD8⁺ T activity due to reduced Gr-1⁻ density by immunohistochemical examination with CD31 antibody (Fig. 4A). The percentage of CD31⁺ positive cells among tumor cells was significantly lower in CC-LR Il17⁻/⁻ mice in comparison to CC-LR Il17⁺/⁺ mice (CC-LR Il17⁻/⁻ 8.9 ± 1.1%, CC-LR Il17⁺/⁺ 4.8 ± 1.2%, P = 0.011), indicating IL-17 promotes angiogenesis in lung adenocarcinoma model (Fig. 4B).

To examine whether IL-17 increases the in vivo growth of tumors by promoting angiogenesis, we evaluated the vascular density by immunohistochemical examination with CD31 antibody (Fig. 4A). The percentage of CD31⁺ positive cells within the tumors were significantly lower in CC-LR Il17⁻/⁻ mice in comparison to CC-LR Il17⁺/⁺ mice (CC-LR Il17⁻/⁻ 6.4 ± 1.0%, CC-LR Il17⁺/⁺ 3.0 ± 1.2%, P = 0.036), indicating that IL-17 promotes angiogenesis in lung adenocarcinoma model (Fig. 4B).

IL-17 is known to stimulate the expression of chemokines and proinflammatory molecules in epithelial cells during inflammation. Therefore, we examined the molecules that are directly regulated by IL-17. Although the expression of Cxcl1 or Csf2 remained similar between CC-LR Il17⁺/⁺ and CC-LR Il17⁻/⁻ mice, Il6, Cxcl2, Ccl2, Il12b, Arg1, Csf3, Mmp7, Mmp12, and Mmp13 (Table S3) were up-regulated in tumor-bearing lung and reduced in CC-LR Il17⁻/⁻ mice in comparison with CC-LR Il17⁺/⁺ animals (Fig. 4C). Compensatory T-cell response in CC-LR Il17⁻/⁻ mice could influence the control of tumor growth; however, IFNγ expression in CD4, CD8 T cells and non-CD4 CD8 T cells were similar in WT and CC-LR Il17⁻/⁻ mice. Therefore, our data suggest that IL-17 is required for tumorogenesis by inducing proinflammatory molecules during the development of lung cancer.

**IL-17 Regulates Gr-1⁺CD11b⁺ Myeloid Cells in Lung Cancer.** To understand why CC-LR Il17⁻/⁻ mice developed fewer adenocarcinomas than CC-LR Il17⁺/⁺ mice at 14 wk of age, we examined cellular infiltrates from lung parenchyma and tumors. Although the BALF of WT mice was mostly composed of alveolar macrophages, CC-LR mice showed elevated number of lymphocytes and macrophages and elevated occurrence of a distinctive Gr-1⁺CD11b⁺ population, which is significantly reduced in CC-LR Il17⁻/⁻ mice (Fig. 4D). The gated Gr-1⁺CD11b⁺ cells in BALF are mostly Ly6G-positive cells (Fig. S4A). Lung parenchyma of normal mice, however, is comprised of mature and terminally differentiated Gr-1⁺CD11b⁺ neutrophils. Cellular analysis of lung parenchyma quantified by immunohistochemical staining of Ki67 (Fig. 4A). The percentages of Ki67⁺positive cells among tumor cells were significantly lower in CC-LR Il17⁻/⁻ mice in comparison to CC-LR Il17⁺/⁺ mice (CC-LR Il17⁻/⁻ 8.9 ± 1.1%, CC-LR Il17⁺/⁺ 4.8 ± 1.2%, P = 0.011), indicating IL-17 promotes tumor cell proliferation directly or indirectly (Fig. 4B).

**Fig. 2.** Increased Th17 cells in inflammation-promoted lung cancer. (A) Cytokines in BALF by ELISA (n ≥ 5 per group). (B) Representative flow cytometry plots of T cells. Lung mononuclear cells were isolated from 14-wk-old WT or CC-LR mice which were challenged with NTHi lysate for 4 wk. Isolated cells were stimulated with PMA/Ionomycin and stained with antibodies to CD4, CD8, CD3e, IL-17, IFNγ, and Foxp3. (C) Frequency of lung CD4 T cells expressing IL-17, IFNγ, and Foxp3. Data are shown as mean ± SEM, *P < 0.05, **P < 0.01 (WT + NTHi, n = 6; CC-LR + NTHi, n = 8).

**Fig. 3.** IL-17 deficiency reduces lung cancer development. (A) Tumor numbers were counted on the lung surface at 14 wk of age in CC-LR mice that spontaneously develop lung adenocarcinoma, compared with the same mice crossed onto IL-17⁻/⁻ or IL-17F-deficient mice, CC-LR Il17⁻/⁻ or CC-LR Il17F⁻/⁻. (B) Total cell number in BALF. Data are shown as mean ± SEM. **P < 0.01. ***P < 0.001 (CC-LR, n = 6; CC-LR Il17⁻/⁻, n = 7; CC-LR Il17F⁻/⁻, n = 5). (C) Representative H&E-stained lung sections from 14-wk-old CC-LR, CC-LR Il17⁻/⁻ and CC-LR Il17F⁻/⁻ mice. (D) Tumor numbers were counted on the lung surface at 14 wk of age in CC-LR mice that are exposed to NTHi starting at age 10 wk weekly for 4 wk. (E) Total cell numbers in BALF. Data are shown as means ± SEM (NTHi + CC-LR, n = 5; NTHi + CC-LR Il17⁻/⁻, n = 8). *P < 0.05. **P < 0.01.
CD11b+ cells. However, when CD8 T cells in CC-LR Il17−/− were depleted using anti-CD8 antibody for 2 wk, we did not observe altered tumor development in CC-LR Il17−/− mice (Fig. 5). In addition, we observed that CC-LR mice that were depleted of CD8 T cells exhibited similar tumor burden as isotype control antibody-treated mice (Fig. 5). Therefore, the cytotoxic activity of CD8 T cells is not attributable to reduced tumor growth in CC-LR mice lacking IL-17, at least in a period of 14 wk we observed.

To investigate the role of tumor-associated Gr-1+CD11b+ cells in the development of lung adenocarcinoma and establish whether IL-17 mediated cellular changes in tumor are in agreement with the immune responses mediated by Gr-1+CD11b+, we depleted Gr-1+ cells by i.p. injection of the RB6.8C5 monoclonal antibody in tumor-bearing mice. Depletion of Gr-1+ cells was confirmed by flow cytometric analysis of BALF and lung (Fig. 6A). Depletion of Gr-1+CD11b+ cells in CC-LR mice resulted in suppression of lung tumor growth (isotype control group, 5.11 ± 1.90; RB6.8C5 treated group, 27.33 ± 6.76, P = 0.042) (Fig. 6B). Tumor cell proliferation (isotype control group, 16.96 ± 2.63%; RB6.8C5-treated group, 8.49 ± 1.92%, P = 0.013) and tumor microvessel density (isotype control group, 10.5 ± 1.5%; RB6.8C5-treated group, 8.49 ± 1.92%, P = 0.042) were also significantly reduced in Gr-1 antibody-treated group (Fig. 6C). In addition, inflammatory molecules induced upon tumor growth were significantly suppressed upon Gr-1 antibody injection (Fig. 6D). Based on the above data, Gr-1+ cells depletion in CC-LR mice considerably recapitulates the phenotype of CC-LR Il17−/− mice. The expression level of IL-17 upon Gr-1 cell depletion was reduced but the expression level of Foxp3 or IFNγ remained similar (Fig. 6D). We also found that administration of a neutralizing antibody to IL-17A in vivo significantly reduced tumor count as well as Gr-1+CD11b+ cell recruitment (Fig. S5). This result not only highlights the potential use of IL-17 blockade in lung cancer patients, but also rules out possible microbiota difference in WT and CC-LR Il17−/− mice accounting for the tumor growth differences. Collectively, our data demonstrated that Gr-1+CD11b+ myeloid cells recruited during activating lung mutation development promote tumor growth and IL-17 orchestrates their recruitment.

**Discussion**

Understanding the developmental process and function of immune response during tumorigenesis is a challenging problem. One of important factors to consider is to use a disease model that resembles the development of tumors in human. The most common molecular changes identified in human lung cancer are K-ras mutations (31). K-ras mutation restrictively expressed in mouse lung epithelial cells led to cellular hyperplasia, adenoma, and eventually adenocarcinoma, resembling human lung cancer development (25, 28). Therefore, this system allows immune response to be shaped against endogenous arising lung tumor and reflects tumor microenvironment during the initiation and early stages of pulmonary adenocarcinoma.

Th17 cells and its signature cytokine, IL-17, have been detected in different types of human cancers (12). Despite relatively well-defined role of Th17 cells in promoting inflammation in autoimmune disease, their function in tumor development has been obscure. In this study, we made an intriguing observation that activating K-ras mutation in lung epithelial cells promotes the recruitment of Th17 cells in tumor tissue. More importantly, our study revealed an indispensable role of IL-17 in tumorigenesis during the early stages of pulmonary adenocarcinoma. Our conclusion is in agreement with the recent studies using spontaneous or chemically induced colorectal cancer models (17, 19, 20) and prostate cancer model with Pten deficiency (32).

The mechanisms underlying protumorigenic role of IL-17, however, were not completely understood. In our study, IL-17 accelerated cancer development at least in part by recruiting myeloid cells and promoting inflammation. A recent study demonstrated that IL-17 promotes tumor resistance to VEGF inhibition due to its ability of Gr-1+CD11b+ cell mobilization and recruitment (33). Il17rc−/− (deficiency of IL-17 in IL-17F and IL-17F) mice crossed with deletion of PTEN in prostate cells showed reduced prostate adenocarcinoma due to reduction in MMP7. MMP7 was induced directly by IL-17 in prostate cells in this study (32). Because IL-17 can induce IL-6 from tumor and stromal cells, STAT3 activation by IL-6 was proposed as one of mechanisms for protumorigenic role of IL-17 (22). In our study, we also observed...
The authors treated CC-LR mice in comparison with CC-LR WT mice in several cancer models. In a colon cancer model adopting Transwell, decreased CD8+ T cells were found in lung adenosarcoma immunostained with Ki67 or CD31. Ki67-positive cells in CC-LR mice were gated in FSC+SSHi. (B) Tumor burden after anti–Gr-1 treatment (control Ig). (C) Representative sections of lung adenosarcoma immunostained with Ki67 or CD31. Ki67-positive cells or CD31 positive cells per 100 tumor cells (circle, control Ig; square, anti–Gr-1). (D) Relative expression of mRNA in whole lungs after anti–Gr-1 treatment in CC-LR mice. Data are expressed as fold increase compared with controls. Data represent means ± SEM (P<0.05, **P<0.01, ***P<0.001).

In contrast, antitumorigenic role of IL-17 has been reported in several cancer models. In a colon cancer model adopting truncated form of adenomatous polyposis coli (APC), IL-17R−/− × APC−/− mice developed more invasive cancer (34), even though fewer polyps and reduced Gr-1CD11b+ myeloid cells were found in IL-17R−/− × APC−/− mice. In other metastasis accompanying models, IL-17 has shown to promote the activation of tumor-specific CD8+ T cells that help to eradicate tumor (21, 23, 35). Despite the increased tumor burden in IL-17R−/− mice, tumor lung of IL-17R−/− mice expressed reduced Gr-1CD11b+ myeloid cells in comparison with WT (23). In transplantation models, CD8+ T cell response initiated upon tumor injection is a critical determinant of tumor elimination, which may outweigh other cellular effects mediated by IL-17. Therefore, the role of IL-17 in tumor development is likely to be dependent on the local tumor microenvironments and the stage of tumor development.

At present, it is unclear which pathways or molecules are responsible downstream of IL-17 that promote the recruitment of myeloid cells. A recent report indicates CXCL1/2 mediates mammary tumor growth and lung metastasis by recruiting CD11b+Ly6G+ granulocytic MDSC population to the tumor site (36). Alveolar epithelial cells transformed by oncogenic K-ras are known to express high level of CXCL1 and CXCL2 (37). Also, oncogenic K-ras-induced GM-CSF is capable of inducing proliferation and differentiation of c-kit+ Lineage− precursors into functional MDSC (38, 39). Because IL-17 can directly act on lung epithelial cells to induce CXCL2 and G-CSF, reduced expression of these molecules in the lungs of IL-17-deficient mice could contribute to decrease in Gr-1CD11b+ cells recruitment. Even though Gr-1CD11b+ cells in tumor lung were capable of suppressing CD8 T-cell activity ex vivo, depletion of CD8 T cells did not lead to enhanced tumor growth in our study. Gr-1CD11b+ cells may directly promote angiogenesis or promote regulatory T cells to enhance tumor in CC-LR mice (5, 40, 41).

Th17 cells can be readily found in a steady state in intestine but efficiently generated and recruited to tissues upon inflammation. Analysis of human lung cancer patients revealed infiltration of Th17 cells (42). We and others have previously used an experimental model of smoke exposure and demonstrated IL-17-deficient mice are resistant to smoke-induced airway inflammation and emphysema (43, 44). Tobacco smoke accounts for the most common causes of lung cancer. Therefore, these studies suggest the link between inflammation in lung caused by smoking, promotion of lung cancer, and involvement of IL-17 and Th17 cells. In our study, CC-LR mice were challenged with NTHi to induce COPD-like inflammation in the lung. Th17 cells were major adaptive immune cells generated in the lung during inflammation, and IL-17 played a pivotal role in promoting tumor in inflammation-accelerated lung cancer model.

Currently, we do not know how lung cancer microenvironment in K-ras mutant mice directs Th17 cell responses. K-ras activation in the bronchiolar epithelium has been associated with promotion of inflammatory response (28). More recent data indicate K-ras activation in pancreas leads to perturbation of multiple metabolic pathways (45). It is possible that inflammatory molecules or metabolites upon K-ras activation in lung epithelium influence the polarization and recruitment of Th17 cells in lung. Also, it is not known whether Th17 cells in CC-LR mice develop in antigen-specific pathway and recognize self or tumor antigen. In NTHi-challenged CC-LR mice, recall response indicates the generation of NTHi-specific T cells. In contrast, we did not detect IL-17 or IFNγ response to tumor lysates. In APC−/− mice, microbial translocation upon epithelial barrier loss directed the generation of Th17 cells, suggesting the possibility of microbiota-specific Th17 cells in colon cancer (19). CC-LR mice represent the early stage of lung cancer; it would be interesting to define the antigens in this model, which could also differ from the antigens of established tumors.

In summary, we have shown that oncogene-driven and inflammation-promoted lung adenosarcoma in mice is associated with increased Th17 cells in the tumor tissue. Our study also demonstrates, to our knowledge, for the first time that inflammation occurs in the lung can accelerate lung cancer through Th17 cells and IL-17 upon oncogenic activation. Because the lung is vulnerable to many airborne infections and environmental insults that lead to various degrees of inflammation, it is important to identify the cellular and molecular pathways of inflammatory diseases, such as COPD, that predispose individuals to lung cancer. Currently, no screening strategies exist that can identify patients with chronic lung inflammation with the potential to develop lung cancer. A large cohort study measuring the level of Th17 cells and IL-17 in patients with chronic lung inflammation and different stages of lung cancer with history of inflammatory diseases may provide causal link indicative of individuals with lung inflammation at higher risk of developing lung cancer. This link would further help setting the basis for future translational studies targeting Th17 cells or IL-17. Although inhibition of IL-17 using neutralizing antibody or Th17 cell development by targeting IL-23 have shown their promising results treating autoimmune diseases in clinical trials, the potential benefits and risks of inhibiting Th17 cells or IL-17 for cancer therapy have not been investigated. Our study establishes that targeting Th17 cells or IL-17 in the early stage of lung cancer and in COPD patients with Th17 cell response may be a promising strategy for controlling inflammation that promotes tumorigenesis.
Isolation of Lung Resident Mononuclear Cells. Lungs were harvested after perfusion with PBS. Lungs were first inflated with 0.1 ml collagenase IV and EDTA for 15 min at 37°C. Single cell suspensions were prepared by mechanical dissociation of lung tissue through a 70-μm nylon mesh. Lung cells were suspended in PBS and layered on L53 Lymphocyte Separation (Medium MP Biomedical). Cells were centrifuged at room temperature for 20 min at 900 × g. Mononuclear cells were harvested from the gradient interface.

Depletion of Gr-1 Cells or CD8 T Cells. For Gr-1 depletion study, 12-wk-old mice were injected with 150 μg of anti-Gr-1 antibody (Clone: RB6-8C5; Bioxcell) or isotype control Ab (Rat IgG2a) i.p. two times per week for 2 wk. Depletion of the cells was monitored by FACS analysis of blood. For CD8 T-cell depletion study, the same experimental schedule as above was performed using anti-CD8 antibody (Clone: 2.43; BioXCell). Additional information is provided in SI Materials and Methods.

ACKNOWLEDGMENTS. We thank Dr. Tyler Jacks for the LSL-K-rasG12D, Francesco DeMayo for the CCSP-cre mice, Jonathan Kurie for the LKR13 cells, and members of the C.D. laboratory for their help and discussion. The work is supported by research grants from the National Institutes of Health (to C.D.), Cancer Prevention Research Institute of Texas (to C.D. and S.H.C.), and Research Scholar Grant (RSG-11-115-01-CNE) from American Cancer Society (to S.J.M.). C.D. is the Olga and Harry Wiess Distinguished University Chair in Cancer Research of the University of Texas MD Anderson Cancer Center.

Methods
Mice. All of the mice were housed in the MD Anderson cancer center animal facility, and all experiments were performed in accordance with relevant institutional and national guidelines and regulations approved by the Animal Studies Committee. CCSPCre (27), LSL-K-rasG12D (28), Il17f−/−, and Il17f+/- mice (46) have been described. C57BL/6 mice were obtained from the Jackson Laboratory and crossed with CC-LR mice. CC-LR WT, CC-LR Il17f−/−, and CC-LR Il17f+/- mice were kept in separate cages.

Assessment of Lung Tumor Burden. Lung surface tumor numbers were counted, and then BALF was obtained by collecting 1 mL of PBS through a tracheostomy cannula. The lungs were prepared for histologic analysis first by perfusion with PBS, and then inflation with 10% phosphate-buffered formalin (pH 7.4) at a 20-cm pressure of H2O. Total leukocyte count was determined using a hemacytometer, and cell populations were determined by cytocentrifugation and Wright–Giemsa staining. BALF was centrifuged at 1,250 × g for 10 min, and supernatants were collected for ELISA.

NTHI Lysate Aerosol Exposure. A lysate of NTHi strain 12 was prepared as described (25). CC-LR mice were exposed to NTHi starting at 10 wk of age for 4 wk.

Supporting Information

Chang et al. 10.1073/pnas.1319051111

SI Methods

Flow Cytometry. For FACS analysis, cells were stained with CD4 (GK1.5), CD8 (53-6.7), γδT (eBioGL3), CD11b (M1/70), and Gr-1 (RB6-8C5). For intracellular cytokine analysis, cells were restimulated with 500 ng/mL ionomycin and 50 ng/mL phorbol myristate acetate (PMA) in the presence of Golgi Stop (BD PharMingen) for 5 h. Cells were then permeabilized with Cytofix/Cytoperm Kit (BD PharMingen) and analyzed for the expression of IL-17A or IFN-γ (BD PharMingen). Staining for intracellular Foxp3 was performed using mouse regulatory T-cell staining kit (eBioscience).

Immunohistochemistry. For immunohistochemistry, tissues were embedded in paraffin. After sectioning, 4-μm sections were rehydrated, blocked with 3% (wt/vol) H2O2, and incubated in antigen retrieval buffer. Sections were then stained using unconjugated primary antibodies, Ki67 (1:200, ab16667, abcam), CD31 (1:10, 550274, BD Biosciences), Phospho-Stat3 (1:300, 9145, Cell Signaling), and HRP-conjugated secondary antibodies.

Q-RTPCR. Tissue samples for RNA analysis were collected and mRNA was isolated by homogenization in TRIzol (Invitrogen) using a tissue homogenizer. RNA was reverse transcribed using Affinity Script Reverse Transcriptase kit (Invitrogen), according to the manufacturer’s instructions. Transcripts were measured by quantitative (Q)-RTPCR on a Real Time PCR system (Biorad). All of the primers used in Q-RTPCR are available in Table S3.

Fig. S1. Expression of IL-17 and Foxp3 was increased in the tumor tissues. (A) Representative flow cytometric plots of lung CD4 T cells from 14-wk-old WT and CC-LR mice. Lung mononuclear cells were restimulated with PMA and ionomycin and stained with IL-17 and Foxp3 antibody. (WT, n = 8; CC-LR, n = 6). (B) Relative expression of mRNA in whole lungs of 14-wk-old WT and CC-LR mice (6–8 per group). Data are expressed as fold increase compared with WT. Data represent means ± SEM *P < 0.05. ***P < 0.001.
Fig. S2. IL-17 promotes chronic obstructive pulmonary disease (COPD)-like inflammation. (A) Total cell numbers in bronchoalveolar lavage fluid (BALF) after 25 wk of nontypeable Haemophilus influenza (NTHi) aerosol exposure (6–7 mice per group). (B) H&E-stained sections from the lungs after 25 wk of NTHi aerosol exposure (C) Airway thickness was measured after 25 wk of challenge (four mice per group, five fields examined per mouse). Airway thickness (Au); ratio of A(t) to A(T). Total airway area, A(T), and airway tissue thickness, A(t), were measured by stereologic analysis by grid overlay to histological image (1). Data represent means ± SEM *P < 0.05.


Fig. S3. Quantification of total tumor area. Tumor size was measured by histological evaluation. Pictures of tumor area were taken at low power magnification, using a 4× or 10× objectives with scale bar. Each point represents data from an individual mouse. **P < 0.01.
Table S1. BALF cell numbers evaluated by cytospin and Wright–Giemsa staining in CC-LR vs. CC-LR Il17−/−

<table>
<thead>
<tr>
<th>Cell type</th>
<th>CC-LR</th>
<th>CC-LR Il17−/−</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytes</td>
<td>0.18 ± 0.02</td>
<td>0.08 ± 0.01</td>
<td>P = 0.003</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>0.09 ± 0.005</td>
<td>0.04 ± 0.006</td>
<td>P = 0.004</td>
</tr>
<tr>
<td>Macrophages</td>
<td>4.72 ± 0.19</td>
<td>2.2 ± 0.32</td>
<td>P &lt; 0.001</td>
</tr>
</tbody>
</table>

Cell number is expressed as ×10⁵ cells.
Table S2. BALF cell numbers evaluated by cytospin and Wright–Giemsa staining in NTHi challenged CC-LR vs. NTHi challenged CC-LR Ii17

<table>
<thead>
<tr>
<th>Cell type</th>
<th>CC-LR</th>
<th>CC-LR Ii17−/−</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytes</td>
<td>0.69 ± 0.34</td>
<td>0.04 ± 0.01</td>
<td>P = 0.003</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>8.7 ± 4.3</td>
<td>4.4 ± 0.5</td>
<td>P = 0.03</td>
</tr>
<tr>
<td>Macrophages</td>
<td>10.9 ± 5.33</td>
<td>5.48 ± 0.64</td>
<td>P = 0.03</td>
</tr>
</tbody>
</table>

Cell number is expressed as ×10⁵ cells.

Table S3. List of Q-RTPCR primers

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin</td>
<td>TGGAAATCTGTGCGATCCATGAAAC</td>
<td>TAAAACGCAAGCTCAAGTAAACCTCGC</td>
</tr>
<tr>
<td>Il6</td>
<td>ACCAGAGAAAATTCTCAATAGGC</td>
<td>TGATGCACCTGCAGAAGACCA</td>
</tr>
<tr>
<td>Csf3</td>
<td>TGCTTAAGTCTCCATGGGCAAA</td>
<td>AGCTTGTAGGTCGCAACAA</td>
</tr>
<tr>
<td>Csf2</td>
<td>TGGAAGCATGTAGAGGGCATCA</td>
<td>GGGGCTTGTAGTGGGCGGATT</td>
</tr>
<tr>
<td>Arg1</td>
<td>TTTTCCACGAGACCCACTTTT</td>
<td>AGAGATTATGCGAGCGCCTT</td>
</tr>
<tr>
<td>Mmp7</td>
<td>CGGCCAATCTGCTCAGGAAAG</td>
<td>GGGAGAATTTTCTCAGCTAGG</td>
</tr>
<tr>
<td>Mmp12</td>
<td>GGAAGCCTTCCTGGAGGTCCAGCCA</td>
<td>CTCTTCGCTACATCACTTCCCTAG</td>
</tr>
<tr>
<td>Mmp13</td>
<td>TTACCACTTCCTGGGAGGAAGAAAACA</td>
<td>GTCTTGGTCGCTCAAGTAGTGA</td>
</tr>
<tr>
<td>Ccl1</td>
<td>CGCTCTCTGTGCGAGCCTTCGCTGCT</td>
<td>AAGCCTCGGACACCTCTGAGT</td>
</tr>
<tr>
<td>Ccl2</td>
<td>TGGCATCTTGAGACGTCG</td>
<td>TCCTCCTTTCGAGGCTAGG</td>
</tr>
<tr>
<td>Cxcl1</td>
<td>CGCTTCTGTCGTCGCAGCCGGTTG</td>
<td>AAGCCTCGGACACCTCTGAGT</td>
</tr>
<tr>
<td>Cxcl2</td>
<td>TGGCTCCTCCAGGAGGAACAGAAG</td>
<td>GGTGCTCATGCGCTGGTGG</td>
</tr>
<tr>
<td>Cxcl3</td>
<td>CTCCAGCAATGAGAGACGACGACGAT</td>
<td>GGTGCTCATGCGCTGGTGG</td>
</tr>
<tr>
<td>Il17</td>
<td>CTCCAGCAATGAGAGACGACGACGAT</td>
<td>GGTGCTCATGCGCTGGTGG</td>
</tr>
<tr>
<td>Foxp3</td>
<td>GGGGCAAATCTGCTGCG</td>
<td>GGTGCTCATGCGCTGGTGG</td>
</tr>
<tr>
<td>Ifng</td>
<td>GATGGTAGCATCTGAGTGGCAGCT</td>
<td>GGGGCAAATCTGCTGCG</td>
</tr>
<tr>
<td>Tbx21</td>
<td>CAACACACCTCCATTGTGCAAAG</td>
<td>TCCCCAAAGCATTGAGACG</td>
</tr>
<tr>
<td>Rorc</td>
<td>GAACGCTCCTCCAGGACAGAAG</td>
<td>GGTGCTCATGCGCTGGTGG</td>
</tr>
<tr>
<td>Ahr</td>
<td>AGCATCAGAGAAACCTTG</td>
<td>GGATTTCCGCTGGTATGCG</td>
</tr>
</tbody>
</table>

Chang et al. www.pnas.org/cgi/content/short/1319051111