Progression of EGFR-Mutant Lung Adenocarcinoma is Driven By Alveolar Macrophages

Don-Hong Wang1, Hyun-Sung Lee2, David Yoon2, Gerald Berry1, Thomas M. Wheeler2, David J. Sugarbaker2, Farrah Kheradmand2, Edgar Engleman1, and Bryan M. Burt2

Abstract

Purpose: Lung adenocarcinomas with mutations in the EGFR have unprecedented initial responses to targeted therapy against the EGFR. Over time, however, these tumors invariably develop resistance to these drugs. We set out to investigate alternative treatment approaches for these tumors.

Experimental Design: To investigate the immunologic underpinnings of EGFR-mutant lung adenocarcinoma, we utilized a bitransgenic mouse model in which a mutant human EGFR gene is selectively expressed in the lungs.

Results: EGFR oncogene–dependent progression and remission of lung adenocarcinoma was respectively dependent upon the expansion and contraction of alveolar macrophages, and the mechanism underlying macrophage expansion was local proliferation. In tumor-bearing mice, alveolar macrophages downregulated surface expression of MHC-II and costimulatory molecules; increased production of CXCL1, CXCL2, IL1 receptor antagonist; and increased phagocytosis. Depletion of alveolar macrophages in tumor-bearing mice resulted in reduction of tumor burden, indicating a critical role for these cells in the development of EGFR-mutant adenocarcinoma. Treatment of mice with EGFR-targeting clinical drugs (erlotinib and cetuximab) resulted in a significant decrease in alveolar macrophages in these mice. An activated alveolar macrophage mRNA signature was dominant in human EGFR-mutant lung adenocarcinomas, and the presence of this alveolar macrophage activation signature was associated with unfavorable survival among patients undergoing resection for EGFR-mutant lung adenocarcinoma.

Conclusions: Because of the inevitability of failure of targeted therapy in EGFR-mutant non-small cell lung cancer (NSCLC), these data suggest that therapeutic strategies targeting alveolar macrophages in EGFR-mutant NSCLC have the potential to mitigate progression and survival in this disease. Clin Cancer Res; 1–11. ©2016 AACR.

Introduction

Non–small cell lung cancer (NSCLC) has an overall 5-year survival rate of only 18% and is the leading cause of cancer-related deaths in the world (1, 2). The EGFR is the cell surface receptor for members of the EGF family of extracellular protein ligands. Mutations that lead to EGFR overexpression or constitutive activation have been found in a number of cancers and are associated with increased cell proliferation, migration, and adhesion (3). Ten to thirty percent of NSCLCs have mutations in exons encoding the tyrosine kinase domain of the EGFR gene, and these tumors represent a unique subset of lung cancer (4, 5). EGFR-mutant NSCLCs display unprecedented response rates of 64% to 82% to targeted molecular therapy with EGFR tyrosine kinase inhibitors (TKI), making these reagents the standard first-line treatment for patients with metastatic EGFR-mutant NSCLC (6). However, even in combination with cytotoxic chemotherapy, less than 5% cure rate is expected because nearly all cancers treated with TKIs will develop resistance resulting in disease progression (7). There is an obvious need to develop new strategies to improve long-term outcomes in this disease.

It has become increasingly clear that failure of the immune system to recognize transformed cells could also drive the initiation and progression of cancer. Accordingly, recognition of aberrant immune responses that promote tumor growth has identified a variety of novel therapeutic targets (8). Tumor-associated macrophages are mononuclear phagocytic cells that are a major cellular component of human NSCLC and their abundance in human NSCLC tumors portends an overall poor survival (9). The importance of tumor-associated macrophages in lung cancer is also supported by mouse data demonstrating that macrophages facilitate the development of carcinogen (urethane)-induced (10) and KRAS-driven mouse lung adenocarcinoma (11). In mice and in humans, macrophages foster tumor progression through angiogenic reprogramming, suppression of antitumor immune responses, and production of soluble mediators that support the proliferation, survival, and invasion of malignant cells (12). Therefore, tumor-associated macrophages represent an attractive target for cancer immune modulation and strategies targeting tumor-associated macrophages are currently being introduced into the clinic by way of clinical trials (13–16).

To understand the immunologic determinants of tumor progression in EGFR-mutant lung adenocarcinoma, we utilized a...
**Translational Relevance**

Despite recent advances in treatment of EGFR-mutant lung cancer, narrow therapeutic indices and frequent acquired resistance limit their overall success rate. We reasoned that inhibition of immunologic pathways that support tumor growth and maintenance in this disease could represent an alternative treatment approach to provide long-lived tumor destruction and surveillance. In this communication, we demonstrate that selective depletion of alveolar macrophages from EGFR-mutant lung tumor-bearing mice results in dramatic reduction of tumor burden indicating that these alveolar macrophages play a critical role in the growth and survival of tumors in this model. In tumors for which targeted therapies are available, there could be an important role for combining immunotherapy-based targeting of tumor-associated macrophages with targeted therapy to improve cancer survival.

**Materials and Methods**

**Animal model**

Animal experiments were performed in accordance with the Institutional Animal Care and Use Committees at Stanford University (Stanford, CA) and Baylor College of Medicine (Houston, TX). We utilized a genetically engineered bitransgenic mouse model of lung adenocarcinoma driven by an organ-specific lepidic histology that progresses to invasive adenocarcinoma (17, 18). Herein, we have identified a novel mechanism by which oncogene-dependent tumor progression and remission, respectively, require the expansion and contraction of alveolar macrophages (AM) in an EGFR-mutant model of NSCLC. Specifically, we show that oncogenic EGFR signaling results in the expansion of an overwhelming number of AMs with a functionally immunosuppressive phenotype, the elimination of which result in markedly decreased tumor burden. Moreover, treatment of EGFR-mutant mice with oncogene-inactivating agents such as erlotinib decreased the frequency of AMs in the lungs, suggesting a previously unappreciated mechanism for erlotinib-mediated tumor suppression in this model. Because of the inevitability of TKI resistance in EGFR-mutant NSCLC, these data suggest that concurrent therapeutic strategies that could reduce AMs in EGFR-mediated lung cancer might provide the potential to abrogate tumor progression and prolong survival.

**Histology and IHC**

Mice were euthanized with a lethal dose of carbon monoxide per institutional guidelines. Lungs were cleared of circulating blood cells by perfusion of the right ventricle with PBS, and whole lungs were excised. For hematoxylin and eosin (H&E) staining, lungs were fixed in 2% paraformaldehyde in PBS overnight at room temperature, and stored at 4°C. Paraffin embedding, sectioning, and H&E staining were performed at Histo-Tec laboratories, and the stained sections were reviewed by board-certified pathologists (G. Berry and T.M. Wheeler). For immunofluorescence microscopy, whole lungs were suspended in OCT embedding compound (Tissue-Tek), immediately frozen on dry ice, and then maintained at −80°C. After cryostat sectioning, the sections were post-fixed in 2% paraformaldehyde and blocked with 10% goat serum. Sections were subsequently stained with anti-mouse F4/80 antibody (Biologend, 123122, 1:300), followed by secondary goat anti-mouse (Life Technologies, A-21235, 1:500), and DAPI was used as nuclear stain. Immunofluorescence images were viewed and captured with a Zeiss LSM700 microscope.

**Flow cytometry and cell sorting**

Lungs were collected and minced into small pieces in 50-mL conical tubes and digested in 1.0 mg/mL collagenase IV (Worthington, LS-004188) and 100 U/mL DNase I (Sigma-Aldrich, D4263) for 20 minutes in 37°C. Supernatants were passed through a 100-μm cell strainer and quenched with RPMI1640 with 10% FCS. Single lung cells were washed in a PBS buffer containing 1% FCS and 2 mmol/L EDTA and red blood cells were lysed using an ammonium chloride–potassium buffer for 1.5 minutes at room temperature. Lung cells were blocked with anti-mouse CD16/32 (Biolegend, 101320) and then stained with antibodies against Ly-6G (Biolegend, 127628), CD11b (Biolegend, 101206), CD11c (Biolegend, 121307), CD40 (Biolegend, 113718), IA-Iα (Biolegend, 115208), CD40 (Biolegend, 124614), CD11b (Biolegend, 101128), CSF-1R (Biolegend, 135510), CD206 (Biolegend, 141712), CD64 (Biolegend, 139306), CD80 (Biolegend, 104718), CD86 (Biolegend, 105020), Annexin-V (Biolegend 640905), and DAPI (Life Technologies, D1306). Anti-human EGFR was purchased from Biologend (352906). Flow cytometric data acquisition was performed on a LSRII flow cytometer (BD Biosciences), and data.
analysis was performed using FlowJo software. AM (CD45^+ F4/80^+ CD11c^+ Ly6G^-) and EGFR-mutant epithelial cells (CD45^- human EGFR^+) were sorted from single lung suspensions of bitransgenic mice fed doxycycline for 7 weeks using a FACS Aria II Cell Sorter (BD Biosciences).

Phagocytosis assay
Single lung cell suspensions were rinsed in 1% BSA/HBSS (Life Technologies), and 200 mL of lung cells (1 x 10^6 cells/mL) were added to triplicate wells 96-well bottom-plates (Corning Costar; Sigma-Aldrich). Fluorescein isothiocyanate (FITC)-dextran (molecular weight 40,000; Sigma, FD40S) was then added for a final concentration of 1.0 mg/mL. Samples were incubated at either 37°C, or 4°C to determine basal uptake of FITC-dextran. Samples were collected at 30, 60, and 120 minutes. The samples were then washed twice with 1% BSA/HBSS at 4°C and stained for cell surface markers for analysis by flow cytometry.

Cytokine assays
AMs and EGFR-mutant epithelial cells were sorted from single lung cell suspensions using a BD FACS Aria II (BD Biosciences) and rinsed with 10% FBS/RPMI1640 (Life Technologies). Two hundred and fifty thousand cells were cultured in 200 mL of RPMI 10% FCS for 24 hours at 37°C. Supernatants were assayed by Luminex (Life Technologies) assays and Proteome Profiler cytokine assays (Mouse Cytokine Array Panel A Kit, R&D Systems) according to the manufacturer’s instructions.

Gene expression data and patient cohorts
DNA microarray data from AMs of human smokers (n = 15) and nonsmokers (n = 15), as well as from murine AMs from two mouse models of emphysema including Integrin-β-6-deficient (Igkb6^−/−) mice and transgenic mice with CC10 promoter-driven overexpression of IL13 (Tg(cc10-IL13)Stat6^+/−) were obtained from the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database (www.ncbi.nlm.nih.gov/geo, accession number GSE2125; ref. 20). DNA microarray data from the lungs of CCSP/EGFR (n = 4), transgene negative (n = 4), and monotransgenic littermate mice (n = 4) fed doxycycline for 3 to 6 months were obtained from the NCBI GEO (GSE17373; ref. 21). We obtained mRNA sequencing data (n = 515) of lung adenocarcinoma from The Cancer Genome Atlas (TCGA) portal (https://gdc-portal.nci.nih.gov/) and the results shown here are in whole or part based upon data generated by the TCGA Research Network (http://cancergenome.nih.gov/). Gene expression data from the tumors of patients undergoing resection for early-stage lung cancer from the Japan National Cancer Center Research Institute (JNCC cohort, n = 226) were obtained from the NCBI GEO (GSE31210; ref. 22).

Statistical analysis of microarray data
Biometric Research Branch (BRB)-Array Tools were used for statistical analysis of the gene expression data (23), and all other statistical analyses were performed in the R language environment (http://www.r-project.org). All gene expression data were generated using the Affymetrix platform (U133 plus 2.0). Raw data from the Affymetrix platform were downloaded from public databases and normalized using a robust multiarray averaging method (24). To predict the class of the independent patient cohort, gene expression data in the training set were combined to form a series of classifiers according to the compound covariate predictor (CCP) algorithm as described in previous publications (25) and the robustness of the classifier was estimated by the misclassification rate determined during leave-one-out cross-validation (LOOCV) of the training set. When applied to the independent validation sets, prognostic significance was estimated by evaluating the differences between Kaplan–Meier plots and log-rank tests between the two predicted subgroups of patients. After LOOCV, the sensitivity and specificity of the prediction models were estimated by the fraction of samples correctly predicted. To enumerate the frequency of AMs in tumor tissues by the AM activation (AMac) mRNA signature, we utilized CIBERSORT (http://cibersort.stanford.edu/), a novel analytic method for "in silico flow cytometry" that accurately estimates the abundances of member leukocyte subsets among a mixed cell population using gene expression data (26).

Results
Oncogenic EGFR initiates expansion of AMs
Histologic sections of lungs from bitransgenic (CCSP/EGFR) mice fed doxycycline for 7 weeks demonstrated an increased percentage of cells expressing the macrophage marker F4/80 compared with control bitransgenic mice fed normal chow (Fig. 1A). Analysis of single lung cell suspensions by flow cytometry confirmed increased relative abundance of AMs in the lungs of mice fed doxycycline. At 4 weeks, 56.1% ± 4.3% (mean ± SE) of CD45^- hemopoietic cells in the lung coexpressed F4/80 and CD11c and were negative for the granulocyte marker Ly-6G, consistent with an AM phenotype (Fig. 1B and C). By 7 weeks, 81.4% ± 1.1% of CD45^- lung cells were AMs. In contrast, in control mice fed normal chow for 7 weeks, AMs comprised 17.6% ± 1.5% of all CD45^- lung cells. When quantified in absolute values, AMs increased from an average of 0.5 million AMs in the lungs of control mice, to 11.6 million AMs in doxycycline-fed mice at 7 weeks. The expansion of AMs paralleled an increase in both lung weight (Fig. 1D), and histologic progression to adenocarcinoma (Fig. 1E and F). Human EGFR expression was detected on CD45^- cells and not on AM (Supplementary Fig. S1A) and doxycycline administration itself did not affect AM number or function (Supplementary Fig. S1B and S1C).

AMs from tumor-bearing mice exhibit an immunosuppressive phenotype and are functionally distinct from steady-state AMs
To understand whether AMs in this model of lung adenocarcinoma are phenotypically altered, AMs from doxycycline-fed mice and control mice were compared using flow cytometry. We found similar expression of several known macrophage markers including CD11b, CD206, and CD64 in AMs isolated from the lungs of doxycycline-fed and control mice. In contrast, we found reduced expression of costimulatory molecules, such as CD40, CD80, and CD86 in AMs from doxycycline-fed bitransgenic (CCSP/EGFR) mice, as well as downregulated MHC-II expression (Fig. 2A), consistent with an alternatively activated, or M2, macrophage phenotype (27). Because the functional properties of macrophages may better distinguish AM subsets, we evaluated AMs from tumor-bearing mice and from control mice, with respect to phagocytosis and cytokine secretion, two functions that characterize macrophage phenotypes. For example, increased phagocytic properties of macrophages have been correlated with an M2 phenotype (28). Thus, AMs from tumor-bearing and
control mice were cultured in the presence of FITC-dextran to evaluate their phagocytosis. As expected, control AMs demonstrated greater phagocytic capability compared with lung DCs (CD45<sup>+</sup>CD11c<sup>+</sup>F4/80<sup>+</sup>Ly6G<sup>−</sup> cells) in control mice and mice fed doxycycline for 7 weeks. When compared with AMs from control mice, AMs from tumor-bearing mice demonstrated increased phagocytosis of FITC-dextran (Fig. 2B). We next evaluated cytokines in the supernatants of 24-hour cultures of sorted AMs from tumor and control mice. Compared with AMs from control mice, AMs from tumor-bearing mice produced greater amounts of the cytokines IL1α and TNFα, of the chemokines CXCL1 and CXCL2, as well as the IL1 receptor antagonist (IL1RA; Fig. 2C). Furthermore, elevated levels of CXCL1 were also detected in the brochoalveolar lavage (BAL) fluid of tumor-bearing mice (Fig. 2D).

Local proliferation underlies AM expansion

We sought to determine whether AM expansion in tumor-bearing mice was caused by infiltration of blood-derived monocyte precursors, or local proliferation of AMs. To deplete peripheral (blood) monocytes, doxycycline-fed bitransgenic CCSP/EGFR mice were injected intraperitoneally every other day for 6 weeks with a neutralizing antibody against CSF-1R. In this model, peripheral blood monocytes (CD45<sup>+</sup>CD11b<sup>+</sup>Ly6G<sup>+</sup>) demonstrated relatively high expression of CSF-1R compared with AMs (Fig. 3A). With this intervention, peripheral blood monocytes were significantly depleted (Fig. 3B). This depletion of peripheral blood monocytes during doxycycline feeding had no effect on lung weight (Fig. 3C), absolute number of AMs (Fig. 3D), phenotype of AMs (Supplementary Fig. S2A), or tumor burden (Supplementary Fig. S2B). To determine the contribution of local macrophage proliferation to the increase of AMs in tumor-bearing mice, we double stained tissue section of lungs from doxycycline-fed and control (no doxycycline) mice for F4/80 and the proliferation marker Ki-67 (Fig. 3E). In doxycycline-fed mice, 44.8% ± 6.1% of AMs expressed Ki-67, whereas in control mice 3.5% ± 8.4% of AMs expressed Ki-67 (Fig. 3F). To investigate whether EGFR-mutant epithelial cells produced soluble factors that could potentiate AM expansion, CD45<sup>+</sup> human EGFR<sup>+</sup> cells were sorted from doxycycline-fed mice. Detected in the supernatants of these cultured cells were GM-CSF, M-CSF, CXCL12, IL1α, and TNFα (Fig. 3G). Taken together, these data indicate that local proliferation of macrophages, not recruitment of blood...
monocytes, is the dominant mechanism underlying the expansion of AMs in tumor-bearing mice.

AMs contribute to the progression of EGFR-mutant lung adenocarcinoma

Given the striking expansion of AMs and the immunosuppressive phenotype of AMs in tumor-bearing mice, we hypothesized that tumor progression in EGFR-mutant NSCLC is driven by AMs. To test this hypothesis, we depleted AMs in doxycycline-fed CCSP/EGFR mice by intratracheal delivery of clodronate-encapsulated liposomes. After 6 weeks, AMs in mice receiving clodronate liposomes were significantly depleted (Fig. 4A), and the lungs of mice receiving clodronate liposomes were significantly reduced in weight compared with the mice treated the same that received control liposomes (Fig. 4B). Furthermore, mice administered clodronate liposomes demonstrated decreased tumor burden. Evaluation of the lungs of clodronate liposome–treated mice revealed a significant reduction in the percentage of lung area that contained preinvasive lepidic adenocarcinoma (Fig. 4C and D). Moreover, whereas 0 of 15 mice treated with clodronate liposomes showed evidence of invasive foci of adenocarcinoma, 2 of 11 mice treated with control liposomes exhibited areas of invasive adenocarcinoma. To eliminate the possibility that clodronate liposomes had a direct effect on the tumor cells in this model, CD45^+/C0 human EGFR^+ cells were sorted from doxycycline-fed mice and cultured in the presence of clodronate-encapsulated liposomes, which did not result in an increase in cell death or apoptosis (Supplementary Fig. S3). To study the effect of AMs on tumor cells, AMs were cocultured with CD45^+/C0 human EGFR^+ cells and these experiments demonstrated that AMs had a protective effect on tumor cell death and apoptosis (Fig. 4E).

Attenuation of oncogenic EGFR results in contraction of AMs

It has been shown in this model that cessation of EGFR signaling results in decreased progression of malignant...
In our hands, lungs from mice fed doxycycline for 7 weeks tripled in weight compared with control mice and returned to normal weight when doxycycline was withdrawn (Fig. 5A). Within 2 weeks of discontinuing doxycycline, the frequency of AMs contracted to levels similar to a control group of mice, suggesting that AM expansion is associated with the expression of mutant EGFR (Fig. 5B). To assess the effect of clinically active inhibitors of human EGFR on the AMs, we treated doxycycline-fed mice with either the tyrosine kinase inhibitor (TKI) erlotinib, or an EGFR-neutralizing antibody, cetuximab. Although cetuximab is particular effective in this L858R-driven mouse model of EGFR-mutant lung adenocarcinoma, its utility in humans is less convincing (30) and this agent was used in our studies as a tool compound. Similar to doxycycline withdrawal, AMs contracted to baseline levels after 2 weeks of drug delivery (Fig. 5C).

Significance in human lung adenocarcinoma

We investigated the clinical relevance of AM in human EGFR-mutant lung adenocarcinoma by constructing an "activated" AM mRNA signature (AMac signature) composed of 72 genes overlapped between AMs from human smokers and mouse AMs from at least one of 2 mouse models of emphysema, and which are differentially expressed compared with human AMs from nonsmokers (GSE2125). Clustering demonstrated two distinct patterns when comparing AMs between smokers and nonsmokers (Fig. 6A). Using CIBERSORT, a novel analytic method for "in silico" flow cytometry that accurately estimates the abundances of member leukocyte subsets among a mixed cell population using gene expression data (26), AMs were enumerated from gene expression profiling data of whole lung from EGFR-mutant adenocarcinoma mice. As expected, AMs comprised a large fraction of immune cells in the lungs of these mice (Fig. 6B). Among 515 human lung adenocarcinoma tumors from the TCGA, enumeration of AMs by CIBERSORT demonstrated higher frequency of AMs within 32 EGFR-mutant tumors compared with 408 EGFR wild-type (WT)/KRAS WT tumors \( (P = 0.036) \) and 75 KRAS-mutant tumors \( (P = 0.073; \text{Fig. 6C}) \). The impact of the AMac signature on overall survival was evaluated in 226 patients with early-stage lung adenocarcinoma undergoing complete surgical resection from the Japanese National Cancer Center (GSE31210). Among 127 patients with an EGFR mutation, patients whose tumors expressed the AMac signature had significantly worse overall survival than those who did not; and among 99 patients with EGFR WT tumors, there was no difference in survival of patients with AMac+ or AMac- tumors (Fig. 6D).

Discussion

EGFR-mutant lung adenocarcinoma is the archetypal "oncogene-addicted" tumor in which dramatic and sustained tumor regression results from specific inactivation of a single oncogene. In patients with human EGFR-mutant lung adenocarcinoma, for example, inhibition of oncogenic EGFR signaling by EGFR-specific tyrosine kinase inhibitors results in clinical
responses that are dramatic, but short lived (7). In fact, essentially all patients with EGFR-mutant lung adenocarcinoma tumors treated with EGFR inhibitors will ultimately progress through treatment. It is therefore necessary to understand the mechanisms underlying oncogene-dependent and -independent tumor progression.

Although oncogene inactivation is associated with cellular senescence and cancer cell death, the precise mechanisms underlying oncogene addiction are obscure and not well understood. Provocatively, several oncogenes have been shown to influence the host immune response including tumor MHC expression or tumor differentiation antigens (31, 32), and cytokine secretion or gene expression (33, 34). Furthermore, it has been demonstrated that an intact immune system, specifically the T-cell compartment, is required for sustained tumor regression following oncogene inactivation in mouse models of lymphoma and leukemia (35), and for potent responses to oncogene-targeted molecular therapy in mouse gastrointestinal stromal tumors (36).

In a mouse model of EGFR-mutant lung adenocarcinoma, we have discovered that oncogenic EGFR signaling results in local expansion of AMs with a distinct phenotype that drives tumor progression. Macrophages are a dynamic and plastic lineage of cells that can be categorized, by phenotype and function, as classically activated (M1) immunostimulatory macrophages that facilitate host responses against foreign pathogens and tumor cells or alternatively activated (M2) immunoregulatory macrophages involved in tissue remodeling, angiogenesis, and facilitation of protumor immune responses (12, 37). Although such extreme forms of polarization are conceptually appealing, these often oversimplified and overlapping macrophage definitions may be limiting within the tumor microenvironment as tumor-associated macrophages (TAM) engage in a wide range of biologic functions that are variable in different tumors (38). For example, in most large-scale transcriptome analysis, macrophages demonstrate a mixed phenotype expressing both M1 and M2 markers (38).

In this genetically engineered mouse model of mutant EGFR lung adenocarcinoma, AMs in tumor-bearing mice were compared with AMs in control mice and demonstrated decreased cell surface expression of MHC-II and costimulatory molecules.

Figure 4.
AMs contribute to the progression of EGFR-mutant lung adenocarcinoma. Frequency of AM (A) and lung weights (B) were measured in mice fed doxycycline for 6 weeks and treated with either empty or clodronate-encapsulated liposomes by an intratracheal route. C, H&E staining of lungs from control mice (non-doxycycline fed), and doxycycline-fed mice receiving either empty or clodronate-encapsulated liposomes are shown. D, Percentage of lung area that was occupied by adenocarcinoma was determined in doxycycline-fed mice receiving either empty or clodronate-encapsulated liposomes. These data are pooled from two separate experiments. E, DAPI and Annexin V expression were evaluated on CD45⁺ human EGFR⁺ cells (CD45⁺ huEGFR⁺) cultured in the presence (1:1 ratio) or absence of AMs from mice fed doxycycline for 7 weeks.
CD40, CD80, and CD86; increased production of CXCL1, CXCL2, and IL1RA; and increased phagocytosis. Taken together, these characteristics can be considered representative of an immunoregulatory macrophage phenotype. Decreased macrophage MHC-II expression (27, 39), increased macrophage IL1RA expression (40, 41), and increased macrophage phagocytic ability (28) have each been associated with an "M2" macrophage phenotype. In addition, AM in EGFR-mutant tumor–bearing mice produced high levels of the chemokines CXCL1 and CXCL2, each of which has been associated with tumor promotion in breast (42), skin (43), and esophageal cancer (44). Specific to NSCLC, an immunoregulatory macrophage population may be present in human EGFR-mutant NSCLC and may have clinical importance. In a study of 88 patients with advanced lung adenocarcinoma who had been treated with an EGFR TKI, one subset of TAM with a CD68+ MMR<sup>+</sup> phenotype was correlated with unfavorable overall survival and with unfavorable responses to TKIs (45).

Our finding that selective depletion of AMs from mutant EGFR tumor–bearing mice results in dramatic reduction of tumor burden indicates that AMs play a critical role in the growth and survival of tumors in this model. Macrophages are an important and abundant cellular component of the tumor stroma in both mouse and human tumors, and in recent years, it has become apparent that nonmalignant cells in the microenvironment provide essential support for the malignant phenotype. In human lung cancer and other tumors, the abundance of macrophages is often correlated with worse cancerspecific and overall survival outcomes (9, 46). In tumors, macrophages can promote many important features of tumor progression including tumor cell motility and invasion, angiogenesis, and stimulation of persistent tumor cell growth (38). Substantial evidence indicates that intratumoral macrophages, rather than being tumoricidal or immunostimulatory, adopt a protumoral phenotype in vivo both in the primary and metastatic sites. These data, together with experimental studies showing inhibition of tumor progression and metastasis by ablation of intratumoral macrophages, suggest that these cells may represent an important therapeutic target for cancer treatment, and indeed these strategies are moving forward in the clinic. For example, there are currently ongoing phase I clinical trials evaluating neutralizing agents against CSF-1R on intratumoral macrophages in patients with metastatic solid tumors (ClinicalTrials.gov NCT01316822 and NCT01346358). In addition, the marine antineoplastic alkaloid, trabectedin, which is used to treat patients with ovarian cancer and soft tissue sarcoma, may specifically target TAMs. In mice, this compound demonstrates potent antitumor immunity via suppression of monocyte recruitment to tumor sites, inhibition of macrophage differentiation, and initiation of apoptosis in tumor macrophages (14, 47). Furthermore, agonistic anti-CD40 antibodies have been used to activate intratumoral macrophages in vivo and result in regression of pancreatic carcinoma in mice and in humans (16, 48).

Our studies of AMs demonstrated that local macrophage turnover was the predominant mechanism by which AMs increased in the lungs of tumor-bearing mice. Whereas intratumoral macrophages can be potentially derived from a circulating monocyte pool that infiltrates tumors differentiate into macrophages (49), local proliferation is another mechanism by which macrophages can potentially repopulate resident tissues including tumors. Lung macrophages, for example, have been shown to repopulate locally throughout adult life, both in steady state and after cell turnover, in a way that is predominantly independent of circulating monocytes (50). In our experiments, AMs comprised approximately 80% of all lung immune cells after 7 weeks of doxycycline-induced oncogenic EGFR signaling. Depletion of circulating monocytes by anti-CSF-1R–depleting antibodies did not affect the frequency of AMs in these mice and AMs in doxycycline-fed mice demonstrated significantly increased nuclear staining of the proliferation protein Ki-67. Notably, where anti-CSF-1R therapy has been used to deplete macrophages in other mouse tumor models (13), this antibody depleted monocytes but not lung macrophages in our model, and this was likely secondary to differential expression of CSF-1R on monocytes and macrophages in these mice.

Although the focus of cancer treatment has evolved over the past two decades from relatively nonspecific cytotoxic agents to selective, mechanism-based therapeutics, these targeted therapeutics are often limited by a narrow therapeutic index and frequent acquired resistance, as is the case for EGFR-mutant lung cancer. Although targeted strategies aim to inhibit crucial molecular pathways necessary for tumor growth and maintenance, immunotherapy stimulates a host immune response aimed at generating long-lived tumor

Figure 5:
Attenuation of oncogenic EGFR results in contraction of AMs. Lung weights (A) and absolute number of AMs (B) from control mice were compared with mice fed doxycycline for 7 weeks, and doxycycline-fed mice who had doxycycline withdrawn for either the last 1 or 2 weeks of this time period. C, Control mice were compared with doxycycline-fed mice treated with erlotinib or cetuximab for the last 1 or 2 weeks of doxycycline administration. These experiments were performed in duplicate. *P < 0.01.
destruction and surveillance. In tumors for which targeted therapies are available, there could be an important role for combining immunotherapy with targeted therapy. Collectively, our data support the hypothesis that AMs play an important role in the progression of EGFR-mutant NSCLC. In this tumor, AMs may therefore be a suitable target for immunotherapy, alone and possibly in combination with EGFR-targeted therapy.

Disclosure of Potential Conflicts of Interest
T.H. Wheeler is a consultant/advisory board member for PathXL and DNA-SeqAlliance. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: D.-H. Wang, H.-S. Lee, B.M. Burt
Development of methodology: D.-H. Wang, H.-S. Lee, G. Berry, B.M. Burt
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D.-H. Wang, H.-S. Lee, D. Yoon, G. Berry, D.J. Sugarbaker, B.M. Burt
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D.-H. Wang, H.-S. Lee, D. Yoon, T.M. Wheeler, F. Kheradmand, B.M. Burt
Writing, review, and/or revision of the manuscript: D.-H. Wang, H.-S. Lee, D. Yoon, D.J. Sugarbaker, F. Kheradmand, E. Engleman, B.M. Burt
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D.-H. Wang, H.-S. Lee, D. Yoon, D.J. Sugarbaker, B.M. Burt

Figure 6.
Clinical significance of AMs in human EGFR-mutant lung adenocarcinoma. An activated AM mRNA signature (AMac signature) comprised of 72 genes overlapped between AMs from human smokers and mouse AMs from at least one of two mouse models of emphysema, and which are differentially expressed compared with human AMs from nonsmokers (GSE2125). A, The signature is demonstrated by two distinct clusters of genes differentially expressed in the AMs of human smokers and nonsmokers, with red representing high expression and green representing low expression. B, CIBERSORT was utilized to enumerate AMs from gene expression profiling data of whole lung from EGFR/CCSP bitransgenic mice, from transgene-negative mice, and from monogentic littermate mice fed doxycycline (GSE31210). C, Among 515 human lung adenocarcinoma tumors from the TCGA, the proportion of activated AMs were estimated by CIBERSORT in tumors with an EGFR mutation (n = 32), in EGFR WT and KRAS WT tumors (n = 408), and in tumors with a KRAS mutation (n = 75). D, The Japanese National Cancer Center cohort (GSE31210) was used to determine the association of the AMac signature and overall survival was evaluated in 127 patients undergoing resection for an EGFR-mutant early-stage lung adenocarcinoma, and in 99 patients with an early-stage EGFR WT lung adenocarcinoma.
Study supervision: H.-S. Lee, B.M. Burt

Other (reviewed microscopic slides and provided grading of specimens): G. Berry

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