ABSTRACT

The success in lung cancer therapy with programmed death (PD)-1 blockade suggests that immune escape mechanisms contribute to lung tumor pathogenesis. We identified a correlation between EGFR pathway activation and a signature of immunosuppression manifested by upregulation of PD-1, PD-L1, CTL antigen-4 (CTLA-4), and multiple tumor-promoting inflammatory cytokines. We observed decreased CTLs and increased markers of T-cell exhaustion in mouse models of EGFR-driven lung cancer. PD-1 antibody blockade improved the survival of mice with EGFR-driven adenocarcinomas by enhancing effector T-cell function and lowering the levels of tumor-promoting cytokines. Expression of mutant EGFR in bronchial epithelial cells induced PD-L1, and PD-L1 expression was reduced by EGFR inhibitors in non–small cell lung cancer cell lines with activated EGFR. These data suggest that oncogenic EGFR signaling remodels the tumor microenvironment to trigger immune escape and mechanistically link treatment response to PD-1 inhibition.

SIGNIFICANCE: We show that autochthonous EGFR-driven lung tumors inhibit antitumor immunity by activating the PD-1/PD-L1 pathway to suppress T-cell function and increase levels of proinflammatory cytokines. These findings indicate that EGFR functions as an oncogene through non–cell-autonomous mechanisms and raise the possibility that other oncogenes may drive immune escape.

INTRODUCTION

Although genomic alterations that provide growth advantages to cancer cells are widely recognized to be essential for malignant transformation, discoveries made over the past dec-
develop both virus-induced and non-pathogen-associated tumors more frequently than immunocompetent individuals (2). Although these observations support the idea that immune mechanisms may suppress tumor development, tumor formation implies successful escape from immune control.

To generate efficient antitumor immune responses while maintaining self-tolerance, host reactions are tightly regulated through a combination of stimulatory and inhibitory signals. As T lymphocytes can recognize antigens derived from all cellular compartments presented in the context of surface MHC molecules, these antitumor effector cells have been the principal focus of cancer immunotherapy (3). CTL antigen-4 (CTLA-4) is a critical negative immune checkpoint that limits the induction of potent CTL responses. Extensive clinical testing of human blocking anti-CTLA-4 monoclonal antibodies (mAb) demonstrated an increase in antitumor immunity, with approximately 20% of patients with metastatic melanoma achieving long-term survival; these substantive clinical benefits resulted in U.S. Food and Drug Administration (FDA) approval of ipilimumab as first- or second-line therapy for advanced melanoma (4). On the basis of these important results, a second negative immune checkpoint mediated through interactions of programmed death (PD)-1 with its ligands PD-L1 and PD-L2 has been investigated as a target for cancer immunotherapy (5). Blocking antibodies against PD-1 or PD-L1 have demonstrated substantial clinical activity in patients with metastatic melanoma, renal cell carcinoma, non-small cell lung cancer (NSCLC), and other tumors (6, 7). Preliminary findings raise the possibility that PD-1 blockade might be less toxic than ipilimumab, although more detailed testing is required.

In the NSCLC clinical trials, only a subset of patients responded to PD-1 blockade, and early studies suggested that PD-L1 (CD274) expression may be a biomarker for therapeutic response to anti–PD-1 antibodies. Although PTEN loss has been associated with increased PD-L1 expression in gliomas (8), it is unknown whether specific genomic subsets of lung tumors use the PD-1 pathway as a mechanism of immune escape. One of the most commonly mutated oncogenes in patients with NSCLC is EGFR. Previous studies have shown that activation of the EGFR receptor (EGFR) pathway may be involved in suppressing the immune response in murine melanoma models either through activating regulatory T cells (Treg; ref. 9) or reducing the levels of the T-cell chemotactant CCL27 (10).

Mutations in EGFR frequently arise in the kinase domain, rendering tumor cells sensitive to EGFR tyrosine kinase inhibitors (TKI). However, despite the initial response, tumors invariably become resistant by acquiring either a secondary point mutation in EGFR (T790M) or additional alterations in other genes that bypass the need for ongoing signaling from the mutated EGFR (11). A major focus in the therapy for EGFR-driven lung cancers is the development of therapeutic strategies that either delay acquired resistance or are effective in the setting of acquired resistance, though success in these areas has been limited to date. On the basis of the findings in the melanoma models and given that EGFR is one of the most commonly mutated oncogenes in NSCLC (12), we analyzed the immune microenvironment and a set of immunosuppressive pathways in EGFR-driven mouse lung tumors.

RESULTS

Activation of the EGFR Pathway in Murine Bronchial Epithelial Cells Leads to an Immunosuppressive Lung Microenvironment

The two most frequently detected EGFR mutations in patients with NSCLC are in the kinase domain: L858R substitution in exon 21 and deletions in a specific amino acid motif in exon 19 (Del19; ref. 11). Similar to the patients carrying these mutations, mouse lung tumors carrying EGFR L858R or Del19 mutations initially respond to treatment with erlotinib (EGFR TKI; ref. 13), followed by the development of resistance through acquired second site mutations (T790M). Separate mouse models carrying both T790M and L858R or Del19 have been characterized (14, 15); although these do not respond to erlotinib therapy, they do respond to the mutant-specific irreversible EGFR inhibitor WZ4002 for several weeks before they acquire resistance through other mechanisms (16). Microarray expression profiling of the mouse T790M/L858R (TL) tumors as compared with controls revealed increased levels of Pd-l1 (Pdcd1), Pd-l2 (Cd274), Cdl4, Il-6, Tgh1, and granulin (Grn) along with ligands for the EGFR (EGFR-mutant vs. WT for the gene set shown $P = 3 \times 10^{-20}$; Fig. 1A). Analysis of microarray data from previously reported datasets showed no significant differences in Pd-l1 and Pd-l2 (Pdcd1lg2) expression among tumors derived from multiple models of EGFR-driven lung adenocarcinoma [L858R, L858R/T790M (TL) and exon 19 deletion/T790M (TD); refs. 17, 18], indicating that EGFR-driven tumors of a variety of EGFR mutations display elevated Pd-l1 and Pd-l2 expression as compared with normal lung (data not shown). We next confirmed the expression of PD-L1 on tumor (CD45 human EGFR$^+$) and associated hematopoietic cells by flow cytometry and immunohistochemistry (IHC) in EGFR-driven mouse lung adenocarcinomas (Fig. 1B and Supplementary Fig. S1).

As an initial step to understanding the basis for compromised antitumor immunity in mice carrying EGFR-driven tumors, we analyzed the tumor microenvironment in comparison with the lungs from littermate controls to characterize the alterations associated with oncogene expression. Tumor-infiltrating T cells displayed a significantly lower CD8$^+$/CD4$^+$ and CD8$^+$Foxp3 expression ratio and elevated expression of PD-1 and Foxp3 as compared with T cells in the normal lung (Fig. 1C). We next analyzed immune cell populations in whole lungs and detected a significant increase in the absolute number of PD-1$^+$ and Foxp3$^+$ T cells in the tumor-bearing lungs, with a majority of Foxp3$^+$ T cells expressing PD-1 (Fig. 1D and E).

To assess whether other T-cell inhibitory pathways were also induced in these tumors, we analyzed expression of CTLA-4, Lag-3, and Tim-3 (3). CTLA-4 was dominantly expressed by Tregs, and Lag-3 and Tim-3 were expressed by only a small percentage of PD-1$^+$ and Foxp3$^+$ T cells (Fig. 1E and Supplementary Fig. S2). These results suggest that the PD-1 pathway and Foxp3$^+$ Tregs may be dominantly involved in suppressing effector T-cell function in this setting. PD-1$^+$–positive T cells exhibited a memory and activation phenotype (ref. 19; Supplementary Fig. S2B), raising the possibility that EGFR-driven tumors may be characterized by host T-cell exhaustion, specifically through upregulation of the PD-1 and PD-L1 interactions. Interestingly, these antitumor immune changes were also detected in the mildly sick (based on tumor burden – lung
weight) mice, suggesting these are early events associated with oncogene expression (Supplementary Fig. S3A–S3D).

Although PD-1 can be expressed not only by T cells, but also by other immune cells including B cells and macrophages after stimulation (20, 21), we were able to confirm expression of PD-1 only in T cells in this model by flow cytometry (Supplementary Fig. S4).

In addition to the T-cell phenotypes, we investigated how EGFR-driven tumors may induce an immunosuppressive microenvironment in the lungs. Levels of a number of immunosuppressive cytokines, growth factors, and chemokines involved in immune cell accumulation were significantly higher in bronchoalveolar lavage fluid (BALF) from tumor-bearing lungs compared with those from normal lungs (Fig. 1F and Supplementary Fig. S5A), which correlated with their mRNA expression levels in tumor-bearing lungs (Fig. 1A). Because soluble factors in BALFs can be produced by tumor cells as well as tumor-infiltrating immune cells, we also compared the immune cell populations between normal and tumor-bearing lungs by flow cytometry (gating strategy described in Supplementary Methods). Among major immune cell types, the numbers of alveolar macrophages were significantly increased in tumor-bearing animals, whereas natural killer (NK) cells were significantly decreased (Fig. 1G) and showed a functionally impaired phenotype (Supplementary Fig. S5B).

**In Vivo Efficacy of PD-1 Antibody Blockade in Mutant EGFR-Driven Murine Lung Cancer Models**

To confirm our findings that EGFR-mutant tumors display elevated PD-L1 levels and a T-cell exhaustion phenotype, and to explore whether this upregulation drives escape from immune surveillance, we tested a rat monoclonal blocking antibody to PD-L1 (Pd-l1, the EGFR ligands eregulin (Ereg), amphiregulin (Areg), and betacellulin (Btc), and the cytokines Tgfβ1, granulocyte (Grn), and Il6. Two- and 4-week time points indicate the time between the induction of the transgene with doxycycline and subsequent euthanasia. EGFR-mutant versus WT for the gene set shown P = 3 × 10⁻²⁰. B, left, surface PD-L1 expression on CD45⁺ hematopoietic cell population and CD45⁺ human EGFR⁺ cells (tumor cells) was evaluated by fluorescence-activated cell sorting (FACS). PD-L1 and isotype control staining are shown with the black and gray filled lines, respectively, for normal lung (NL) and tumor-bearing lung (TBL) with either microscopic disease or macroscopic nodules. Right, representative images from the lungs of Del19, TD, and TL mice stained for hematoxylin and eosin (H&E) and PD-L1. Scale bars show 100 μm for all panels. C, CD8⁺/CD4⁺ and CD8⁺/Foxp3⁺ ratios and PD-1⁻ and Foxp3⁺-positive frequencies in total CD3⁺ T cells from NL and tumor (T) from TL mice: n = 4; *, **, P < 0.001; ***, P < 0.0001. D, lung weights of control mice and mice carrying tumors driven by Del19, TD, or TL. Quantitative analysis of PD-1⁻ and Foxp3⁺-positive T cells (NL and TL: n = 4; NL and Del, NL and TD: n = 6). *, P < 0.05 (NL vs. TBL for each group). PD-1⁻, PD-1⁻Foxp3⁺, and Foxp3⁺. E, coexpression of immunosuppressive receptors; Foxp3, PD-1, LAG-3, and Tim-3 in CD3⁺ T cells. **, concentration of cytokines IL-6, TGF-β, progranulin (PGRN), VEGF, GM-CSF, and Chemokine (C-C motif) ligand 2 (CCL2) in BALFs (bronchoalveolar lavage fluid) from NL (white bars) and TBL from TL mice (black bars, NL and TL: n = 6). NL versus TBL for all cytokines, P < 0.02. G, immune cell populations; T cell, B cell, NK cell, granulocytes (Gr), alveolar macrophages (AM), and mixed populations (CD11b⁺F4/80⁺ population; the method to identify each population is shown in Supplementary Methods) in NL and TBL (NL and TL: n = 4); *, P < 0.05. GM-CSF, granulocyte macrophage colony-stimulating factor.
relevant doses of anti–PD-1 mAb [200 μg (~8–10 mg/kg) three times a week], we detected a reduction in tumor growth in all of the EGFR-mutant mouse models by MRI (Fig. 2A and B) and increased apoptosis measured by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) and cleaved caspase-3 staining in TD mice (Fig. 2E). Del19 mice showed the greatest tumor volume reductions (50%–60% of the baseline tumor volume after 4 weeks of therapy; Fig. 2B). TL mice showed a modest tumor shrinkage response but exhibited slowed tumor growth as compared with untreated mice (Fig. 2B). Unlike mice with EGFR-driven tumors, transgenic mice with KRAS-driven tumors did not show any significant response to the treatment with anti–PD-1 antibody (Supplementary Fig. S6A–S6C), despite elevated PD-L1 expression (Supplementary Fig. S7A and S7C), suggesting that factors in addition to PD-L1 influence the therapeutic activity of PD-1 antibody blockade. We also observed significantly increased survival with treatment in all three of the EGFR-mutant mouse models (median survival treated vs. untreated, respectively: Del19 16.5 vs. 9 weeks, P < 0.0001; TD 23.5 vs. 16, P = 0.0005; TL 23.5 vs. 16.5, P < 0.0001; Fig. 2E).

**Figure 2.** In vivo efficacy of PD-1 antibody blockade in EGFR-mutant murine lung cancer models. The antitumor effects of anti–PD-1 antibodies in mouse models of EGFR-driven lung cancers (A–E), A, tumor volume changes by MRI at varying time points; baseline, 2, and 4 weeks after treatment of the indicated genotypes of mice. “H” indicates location of the heart. B, quantification of tumor volume changes as compared with baseline tumor volumes in the mice that were either treated with anti–PD-1 antibody (aPD-1 t.) or left untreated (Unt.). C, representative images of lung sections from tumor-bearing mice (TD) that were either treated with anti–PD-1 antibody for 1 week or left untreated. Sections were stained for H&E, TUNEL, and cleaved caspase-3 staining, respectively. Data points indicate total positive signal per tumor field. For TUNEL, n = 3 for untreated and n = 4 for PD-1–treated mice; for cleaved caspase-3, n = 6 for untreated and n = 3 for PD-1–treated mice. E, Kaplan-Meier survival analysis of the anti–PD-1 antibody treated or untreated groups. *P < 0.05; **P < 0.0001; TL 23.5 vs. 16.5, P = 0.0008; TD 25 vs. 16, P < 0.0001; Del19 16.5 vs. 9 weeks, P < 0.001; TD 23.5 vs. 16, P = 0.0005; TL 23.5 vs. 16.5, P < 0.0001; Fig. 2E).

**Anti–PD-1 Antibody Binds to Activated T Cells and Improves Effector Function**

On the basis of these findings, we explored how PD-1 blockade impacts the characteristics of host T cells and other immunosuppressive factors, including cytokine production and accumulation of tumor-associated macrophages in EGFR-driven lung adenocarcinomas. Severely sick mice (based on tumor burden as determined by right lobe weights) from the two EGFR models, Del19 and TD, which showed more dramatic responses to PD-1 blockade treatment, were treated with a PD-1–blocking antibody for 1 week, and then tumor-bearing lungs were harvested along with lungs from untreated severely sick mice (Fig. 3A). Given that we used a rat immunoglobulin G 2a (IgG2a) therapeutic antibody (clone 29F.1A12), we stained lung T cells with a secondary anti-rat IgG2a antibody as well as the same anti-PD-1 antibody used for treatment to differentiate the T-cell population bound or unbound by the therapeutic antibody. The therapeutic antibody was bound to almost all of the PD-1–expressing CD4+ and CD8+ T cells (Fig. 3B and Supplementary Fig. S8A). After confirming efficient target engagement, we next analyzed the phenotypic changes in CD4+ and
Representative flow cytometry results of PD-1 body on days 0, 3, 5, and 8 (four doses), and then at day 9 mice were sacrificed for analysis. B, anti–PD-1 antibodies after tumor burden was confirmed by MRI imaging. Each group was treated either with isotype control (untreated) or anti–PD-1 antibody on days 0, 3, 5, and 8 (four doses), and then at day 9 mice were sacrificed for analysis. B, representative flow cytometry results of PD-1 μg (therapeutic anti–PD-1 antibody binding) in CD4+ and CD8+ T cells, anti–PD-1 antibody–treated mouse (+ aPD-1), control antibody–treated mouse (- aPD-1). C, changes in total T-cell (CD3+/CD4+), CD8+ T-cells, and Tregs, and ratios of CD8+/CD4+ and CD8+/Treg after PD-1 blockade. D, enhancement of effector T-cell function (IFN-γ production) by PD-1 antibody blockade. E, CD3 IHC (top) and quantification of intratumoral CD3+ cells per high-power field in untreated and PD-1 antibody–treated tumors (bottom). Scale bars indicate 25 μm for all panels. Each point on the graph represents counts from single tumor nodule. For del19, n = 2 for untreated and n = 5 for anti–PD-1 antibody–treated mice. For TD, n = 4 for untreated and n = 5 for anti–PD-1 antibody–treated mice. P = 0.01 for both CD3 graphs. F, concentration of the cytokines IL-6, TGF-β1, and PGRN in BALFs. G, absolute number of alveolar macrophages in lungs from Del19 and TD mice. For all bar graphs in this figure, Del19 (untreated and treated: n = 6 and n = 7) and TD (untreated and treated: n = 6 and n = 6). P < 0.05.

**Figure 3.** Anti–PD-1 antibody binds to activated T cells and improves effector function. A, schematic of the short-tern in vivo treatment of mice with anti–PD-1 antibodies after tumor burden was confirmed by MRI imaging. Each group was treated either with isotype control (untreated) or anti–PD-1 antibody on days 0, 3, 5, and 8 (four doses), and then at day 9 mice were sacrificed for analysis. B, representative flow cytometry results of PD-1 μg (therapeutic anti–PD-1 antibody binding) in CD4+ and CD8+ T cells, anti–PD-1 antibody–treated mouse (+ aPD-1), control antibody–treated mouse (- aPD-1). C, changes in total T-cell (CD3+/CD4+), CD8+ T-cells, and Tregs, and ratios of CD8+/CD4+ and CD8+/Treg after PD-1 blockade. D, enhancement of effector T-cell function (IFN-γ production) by PD-1 antibody blockade. E, CD3 IHC (top) and quantification of intratumoral CD3+ cells per high-power field in untreated and PD-1 antibody–treated tumors (bottom). Scale bars indicate 25 μm for all panels. Each point on the graph represents counts from single tumor nodule. For del19, n = 2 for untreated and n = 5 for anti–PD-1 antibody–treated mice. For TD, n = 4 for untreated and n = 5 for anti–PD-1 antibody–treated mice. P = 0.01 for both CD3 graphs. F, concentration of the cytokines IL-6, TGF-β1, and PGRN in BALFs. G, absolute number of alveolar macrophages in lungs from Del19 and TD mice. For all bar graphs in this figure, Del19 (untreated and treated: n = 6 and n = 7) and TD (untreated and treated: n = 6 and n = 6). P < 0.05.

**Figure 4.** EGFR Pathway Activation in Human Bronchial Epithelial Cells Induces PD-L1 Expression

To broaden our findings that PD-L1/2 expression is upregulated in response to EGFR-driven oncogenic signals in mice, we compared PD-L1 and PD-L2 expression in patient-derived established NSCLC cell lines (23), with a particular focus on lines with EGFR and KRAS mutations. EGFR and KRAS
mutations are the two most prevalent drivers of lung adenocarcinomas, and tumors of these genotypes display distinct natural histories and treatment response. We observed a significant correlation among PD-L1/2 expression with expression of EGFR and its ligands, markers of EGFR pathway activation (P values for individual genes are shown; combined P < 10^{-15}, Fig. 4A). We observed a nonsignificant trend toward increased levels of PD-L1 in EGFR-mutant lines compared with KRAS-mutant lines, though the number of available cell lines with an EGFR mutation for this comparison was small (Supplementary Fig. S11A). High PD-L1 expression at the protein level was confirmed in the six EGFR-mutant lines by flow cytometry (Fig. 4 and Supplementary Fig. S11B). We also observed a similar result in an analysis of previously reported microarray data from patients with lung adenocarcinoma (24), in which there was a significant correlation among expression of EGFR and its ligands and PD-L1 expression (P < 10^{-15}; data not shown).

To test whether ectopic expression of mutant EGFR is able to induce PD-L1 expression, we stably expressed mutated EGFR (TD) in immortalized bronchial epithelial cells (BEAS2B). Expression of the mutated EGFR caused an increase in PD-L1 levels by both real-time PCR and flow cytometry in contrast to expression of KRAS<sup>G12V</sup>, which did not induce PD-L1 (Fig. 4B). This suggests that oncogenic EGFR signaling can drive PD-L1 upregulation. Given that expression profiling of tumors suggested that the EGFR signaling pathway may positively regulate expression of PD-L1 ligands, we next tested the EGFR pathway dependency of PD-L1 expression across NSCLC cell lines. First, we evaluated the levels of PD-L1 in EGFR-mutant cell lines after treatment with sublethal doses of the EGFR TKI gefitinib. Flow cytometry analysis showed a clear reduction of PD-L1 protein expression, independent of effects on cell viability. In addition to the gefitinib-sensitive EGFR-mutated lines, we also treated the gefitinib-resistant H1975 and PC-9R cell lines, which harbor an EGFR T790M mutation, with the irreversible mutant-selective EGFR TKI WZ4002 (15). WZ4002, but not gefitinib, decreased PD-L1 levels in H1975 and PC-9R cell lines (Fig. 4D and Supplementary Fig. S11C), confirming a correlation among PD-L1 levels and dependence on EGFR signaling. Although EGFR mutations predict EGFR TKI sensitivity, some EGFR WT cell lines also are sensitive to EGFR TKIs due to activation of the EGFR pathway by overexpression of EGFR or by increased production of EGFR ligands. Treatment of H358 cells, which have been previously shown to display increased EGFR signaling (25), with gefitinib resulted in PD-L1 downregulation (Fig. 4E). These findings suggest that EGFR pathway activation independent of EGFR

Figure 4. EGFR pathway activation in human bronchial epithelial cells induces PD-L1 expression. A, microarray expression profiling analysis of established cell lines from human patients with NSCLC. Black and red bars indicate identified KRAS or EGFR mutations, respectively. 1GDI-α, MET, proto-oncogene (MET), heparin-binding EGF-like growth factor (HBEGF), EREG, and BTC are EGFR ligands. B, PD-L1 upregulation in BEAS-2B bronchial epithelial cell lines transduced with retroviral encoding KRAS (G12V) or EGFR mutation (T790M-Del19), as assessed by quantitative PCR (qPCR) and flow cytometry (C–E). Reduction of PD-L1 expression in NSCLC cell lines 72 hours after EGFR TKI treatment at the indicated concentrations (in the absence of drug-induced apoptosis). C, EGFR-Del19 mutant PC-9 and HCC827 NSCLCs. D, gefitinib-resistant H1975 NSCLC. E, EGFR WT KRAS-mutant H358 NSCLC. Representative results from three independent experiments are shown. F, sections of formalin-fixed patient tumors carrying EGFR mutations stained with H&E or PD-L1. Top, high expression on tumor cell membrane; middle, low expression on membrane; bottom, expression on macrophages. Scale bars show 100 μm. MFI, median fluorescence intensity; iso, isotype control; DMSO, dimethyl sulfoxide.
mutation may also induce the expression of PD-L1. In addition to these studies of cell lines, we confirmed PD-L1 expression at the protein level by IHC on tumor biopsy samples obtained from patients; of the 12 EGFR-mutant lung tumors we studied, nine stained positive for PD-L1 in the tumor and/or myeloid cells (Fig. 4F and Supplementary Table S1).

**DISCUSSION**

We have demonstrated that activation of the EGFR pathway induces PD-L1 expression and other immunosuppressive factors to accomplish evasion of the host antitumor immune response. This role of EGFR signaling seems to be independent of its effects on cell proliferation and survival, suggesting an active role for the EGFR oncogene in remodeling the immune microenvironment. Pharmacologic blockade of the PD-1 pathway in vitro using EGFR TKIs reduced PD-L1 expression. Blocking the PD-1 pathway in EGFR-mutant genetically engineered mouse models resulted in tumor reduction and significantly increased overall survival. Activation of the EGFR pathway enhances susceptibility of the lung tumors to PD-1 blockade. Because PD-L1 is expressed not only by tumor cells but also macrophages and other cells of hematopoietic origin, our results suggest that the combination of PD-1 blockade with EGFR TKIs may be a promising therapeutic strategy to extend the duration of treatment response and delay development of resistance.

Multiple clinical studies have demonstrated clinical responses to anti–PD-1- and PD-L1–blocking antibodies in patients with lung cancer (6, 7), though biomarkers associated with treatment response remain poorly understood. Preliminary results suggest that PD-L1 expression might be associated with a higher likelihood of response to PD-1 blockade, although not all PD-L1–positive tumors are sensitive (7). Similarly, we found that although both EGFR and KRAS mouse tumors expressed PD-L1, only EGFR-driven models responded to the PD-1 blockade. Although the EGFR transgenes used in these murine models are derived from human cDNA sequences, genetic analysis of human NSCLCs typically reveals the presence of large numbers of somatically mutated proteins that might be targets for antitumor T cells (26). Future studies will investigate the antigens triggering T-cell responses in the murine EGFR-driven lung cancer models.

A growing body of evidence suggests that several oncogenes may directly influence the tumor microenvironment through regulation of the expression of soluble ligands and cytokines (27). Secretion of these factors may act in paracrine to stimulate and transform neighboring cells, while recruiting myeloid-derived suppressor cells and Tregs, which are associated with poor prognosis, in contrast to the tumor-infiltrating cytotoxic lymphocytes, which are associated with a better prognosis (28). EGFR-mutant mice exhibited an impaired immune response that involved not only by inducing PD-L1 expression, but also through the production of cytokines and immunosuppressive cells, such as Tregs and macrophages. PD-1 blockade reduces tumor burden by both eliminating tumor cells as well as reducing both the levels of tumor-promoting cytokines and the numbers of immunosuppressive cells. These findings also suggest that a part of the mechanism of action of TKIs in NSCLCs may involve reversing the EGFR pathway–driven immunosuppression in the tumor microenvironment in addition to the well-understood effects on EGFR-driven intracellular signaling.

In murine melanoma models and possibly patients with melanoma, the combination of anti–PD-1 and anti–CTLA-4 antibodies may be more effective than either agent alone due to the complementary functional roles of these two negative immune checkpoints (34, 35). PD-1 blockade in the EGFR-driven mouse lung cancer models did not alter the numbers of Tregs that express high levels of CTLA-4, suggesting a rationale for combined antibody treatment. Additional work is required to explore this possibility and other potential combinatorial therapies, and to delineate the differences in treatment response among the varying EGFR mutations and other oncogene-activated models.

**METHODS**

**Microarray Data Analysis**

For gene expression analysis of NSCLC cell lines, Robust Multi-chip Average normalized expression data were downloaded from the cancer cell line encyclopedia (www.broadinstitute.org/ccle). Expression data from WT and EGFR transgenic mice were obtained from a previous study (18) and converted into log2 values. Pearson correlation coefficient P-values were calculated by comparing expression values for each transcript over all samples to either PD-L1 or PD-1 expression.

**Cell Line Experiments**

BEAS2B cells (ATTC # CRL9609) were grown in bronchial epithelial cell basal medium (Lonza; #CC-3170) and maintained with HEPES buffer solution, trypsin, and trypsin-neutralizing solution (Lonza; #CC-5034). Mutations in EGFR or KRAS were introduced and cloned into the PLPCX vector (Addgene). Clones stably expressing the mutant EGFR or KRAS were selected with puromycin (2 μg/ml) for 3 days. Other cell lines were purchased from the American Type Culture Collection. Genotypes of the patient-derived NSCLC cells are as follows: PC9-del 19, HCC827-del 19, H1975-L858R/T790M, PC9-del 19/T790M. All cell lines were cultured in RPMI-1640 (Corning) supplemented with 10% heat-inactivated FBS, 100 μg/ml penicillin, 100 μg/ml streptomycin and 10 mmol/L HEPES. For PD-L1 expression analysis, untreated cells and gefitinib or WZ4002-treated cells were stained with anti–PD-L1 antibody (29E.2.A3) and isotype control (BioLegend) and then stained with Annexin V and 7-aminoactinomycin D (eBioscience); PD-L1 levels were determined for the Annexin V and 7-AAD double-negative population using a BD FACSCanto II flow cytometer equipped with Diva software (BD Biosciences). The final analysis and graphical output were performed using FlowJo (TreeStar). Treatment doses that did not compromise cell survival were determined with a CellTiter-Glo Luminescence Cell Viability Assay (Promega) after 72 hours.

**Real-Time PCR**

Total RNA was extracted using TRIzol (Invitrogen; #15565018) followed by RNA cleanup (Qiagen; #74204). cDNA were prepared from total RNA prep using the High-Capacity RNA-cDNA Kit (Invitrogen; #43774774). Real-time assays were conducted using TaqMan real-time probes (Invitrogen) for human PD-L1/CD274 (Hs01125301_m1) and GUSB (Hs00939627_m1) using 40 ng cDNA.
Triplcates were run for each sample. GUSB was used as internal control and ΔΔCt method was used for relative mRNA calculations.

**Mouse Husbandry and Breeding**

All EGFR transgenic mice carrying tetracycline-inducible human EGFR cDNA were previously generated, crossed with CC10-RTTA mice expressing reverse tetracycline activator from lung Clara cell CC10 promoter as previously described (13–15), and maintained in mixed (C57Bl/6, FVB, and S129) background. Double-positive progeny were bred with doxycycline diet starting at 5 to 6 weeks of age for the induction of tumors and maintained on doxycycline throughout the study. All breedings and in vivo experiments were performed with the approval of the Dana-Farber Cancer Institute (Boston, MA) Animal Care and Use Committee.

**Antibody Dosing**

Mice received rat anti-PD-1 mAb (clone 29F.1A12) by intraperitoneal injections [200 μg in PBS per dose (8–10 mg/kg), three times a week], as described previously (36). Control mice received similar injections of 200 μg of rat IgG2a isotype control in PBS (BioXcell).

**MRI Tumor Volume Quantification and Survival**

Tumor volume quantifications were performed using the 3D-Slicer software as described in detail in Supplementary Methods. Survival curves were generated by pooling animals that were sacrificed because of heavy tumor burden or were otherwise found dead.

**BALF Collection and Cytokine Measurement**

One milliliter of PBS was injected into the trachea to induce lung washings, which were then aspirated and frozen. Cytokine concentrations in serum and BALFs were measured with ELISA kits for mouse IL-1α, IL-1β, TNF-α, IFN-γ, IL-6, IL-12, IL-18, VEGF, GM-CSF (granulocyte macrophage colony-stimulating factor), MFG-E8, CCL17 (R&D Systems), CCL2, CCL5, and CXCL10 (eBioscience).

**Histology and IHC**

Mice were classified at euthanasia into mild or severe pulmonary pathology based on lung weights: severe (s), total right lung weight ≥ 650 mg; mild (m), total right lung weight < 650 mg. Lungs were inflated with 10% formalin and embedded in paraffin. Sections were stained using anti–PD-L1 antibody clone “7G11” as described previously (37). Cells stained positive for the indicated markers were counted using the automated immunostainer (Ventana) on patient #17-141). Cells stained positive for the indicated markers were counted using the automated immunostainer (Ventana) on patient #17-141).

**Statistical Analysis**

All numerical data are presented as mean ± SD. Data were analyzed using unpaired two-tailed Student’s t test. P values for the survival curves have been calculated using a log-rank test.

**Disclosure of Potential Conflicts of Interest**

M.D. Wilkerson is employed as a consultant at Cancer Therapeutics Innovation Group and GeneCentric. P.A. Janne is a consultant/advisory board member of Boehringer-Ingelheim, Roche, Abbott, AstraZeneca, Pfizer, Sanoﬁ, Chugai Pharmaceuticals, and Clovis Oncology, and has given expert testimony for LabCorp. G.J. Freeman has ownership interest (including patents) in Bristol-Myers Squibb, Roche, Merck, EMD-Serrono, Boehringer-Ingelheim, Amplimmune, and CoStim Pharmaceuticals, and is a consultant/advisory board member of CoStim Pharmaceuticals. G. Dranoff has received commercial research grants from Bristol-Myers Squibb and Novartis, and is a consultant/advisory board member of Novartis, Merck, and Genentech. No potential conflicts of interest were disclosed by the other authors.

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