The Combined Effect of FGFR Inhibition and PD-1 Blockade Promotes Tumor-Intrinsic Induction of Antitumor Immunity

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Abstract

The success of targeted or immune therapies is often hampered by the emergence of resistance and/or clinical benefit in only a subset of patients. We hypothesized that combining targeted therapy with immune modulation would show enhanced antitumor responses. Here, we explored the combination potential of erdafitinib, a fibroblast growth factor receptor (FGFR) inhibitor under clinical development, with PD-1 blockade in an autochthonous FGFR2K660N/p53mut lung cancer mouse model. Erdafitinib monotherapy treatment resulted in substantial tumor control but no significant survival benefit. Although anti–PD-1 alone was ineffective, the erdafitinib and anti–PD-1 combination induced significant tumor regression and improved survival. For both erdafitinib monotherapy and combination treatments, tumor control was accompanied by tumor-intrinsic, FGFR pathway inhibition, increased T-cell infiltration, decreased regulatory T cells, and downregulation of PD-L1 expression on tumor cells. These effects were not observed in a KRASG12C-mutant genetically engineered mouse model, which is insensitive to FGFR inhibition, indicating that the immune changes mediated by erdafitinib may be initiated as a consequence of tumor cell killing. A decreased fraction of tumor-associated macrophages also occurred but only in combination-treated tumors. Treatment with erdafitinib decreased T-cell receptor (TCR) clonality, reflecting a broadening of the TCR repertoire induced by tumor cell death, whereas combination with anti–PD-1 led to increased TCR clonality, suggesting a more focused antitumor T-cell response. Our results showed that the combination of erdafitinib and anti–PD-1 drives expansion of T-cell clones and immunologic changes in the tumor microenvironment to support enhanced antitumor immunity and survival.

Introduction

Non–small cell lung cancer (NSCLC) is the leading cause of cancer-related mortality globally (1). Targeted agents direct-
and subsequent large-scale tumor sequencing initiatives revealed frequent dysregulation of the FGFR family in NSCLC and other tumor types (5, 6). Preclinical analysis of these genetic alterations in model systems has revealed these mutations to be potent oncogenes in vitro and in vivo (7, 8).

The high frequency of FGFR genetic alterations in human cancers provides a compelling rationale to advance specific FGFR inhibitors for patients with FGFR genetic aberrations. Erdafitinib is a selective pan-FGFR inhibitor that has demonstrated preclinically potent antitumor activity in patient-derived xenograft models driven by FGFR mutation (9). Erdafitinib is currently being examined in clinical studies in patients with FGFR-activating mutations and has demonstrated clinical benefit in bladder cancer patients with FGFR point mutations or gene rearrangements (10, 11). However, similar to other targeted agents, it is important to examine combination strategies that can prolong clinical benefit in patients with FGFR alterations.

Cancer immunotherapies, such as those targeting the immune checkpoint PD-1, have revolutionized cancer treatment across a variety of tumor types, including NSCLC. However, only a subset of patients benefits from PD-1/PD-L1 checkpoint blockade. Lack of response to immunotherapy is characterized by several factors, such as a noninflamed tumor microenvironment (TME) with limited infiltrating T cells and/or the presence of immunosuppressive cell types. Tumor genetics can also influence response to checkpoint inhibitors, as evidenced by reports showing that activation of the β-catenin/Wnt pathway renders a non-T cell–inflamed TME (12). Patients with tumors carrying low mutational burden but in concert with driver pathway mutations such as ALK or EGFR may benefit much less from anti–PD-1 or anti–PD-L1 therapy due to a lack of an inflammatory microenvironment and CD8+ T cells that recognize tumor neoantigens (13–15). Overall, these data suggest that defects in antitumor immunity need to be addressed to extend immunotherapy benefit in patients with these driver pathway mutations (16). Here, we specifically explored the contribution of tumor-intrinsic targeting of FGFR driver pathway mutations and its impact on remodeling the TME in an autochthonous lung cancer FGFR2-driven mouse model with low mutational burden (17). Our data showed the key role of FGFR inhibition on remodeling the immune microenvironment of tumors, especially inducing new T-cell responses, which in turn act in concert with anti–PD-1 to promote antitumor immunity.

These results provide a rationale for the combined clinical testing of erdafitinib and PD-1 blockade in patients with FGFR-altered tumors.

Materials and Methods

Genetically engineered mouse models

The FGFR2-mutant transgenic mouse strain conditionally expressing human FGFR2 with the kinase domain activating mutation C1014Y [CF4GFR2_Hsd(CAG-FGFR2_HsdK660N)Kika (FGFR2K660N), Kwok-Kin Wong Lab, New York, NY] and its compound strain, KRASG12C-mutant transgenic mouse strain conditionally expressing human KRAS with the KRASG12C oncogenic mutation (19) were monitored for tumor development by MRI after intratracheal induction with Adeno-cre virus (1 × 10^6 pfu/mouse, University of Iowa) when mice were 7 to 10 weeks of age. Mice with lung tumors confirmed by MRI were randomized into four treatment groups for short-term pharmacodynamic studies as described in Materials and Methods.

Treatment studies

Mice with lung tumors confirmed by MRI were randomized into four treatment groups: control, anti–PD-1 (10 mg/kg, Bio X Cell, RMP1-14), erdafitinib [Janssen Pharmaceuticals, 12.5 mg/kg in 20% 2-hydroxypropyl-ß-cyclodextrin (HPßCD), pH 4.0], and combination of erdafitinib and anti–PD-1. Control animals were treated with 20% HPßCD, pH 4.0, and rat IgG2A isotype (10 mg/kg, Bio X Cell, 2A5). For long-term survival studies, mice were dosed intraperitoneally twice a week with anti–PD-1 or rat IgG2A isotype and orally twice a day with erdafitinib for 4 weeks. After treatment initiation, mice were imaged at weeks 2, 4, and 6 and monitored for survival endpoints for up to 30 weeks. For short-term pharmacodynamic studies, mice were dosed intraperitoneally every other day with either anti–PD-1 or rat IgG2A isotype and orally twice a day with erdafitinib for a week. Baseline blood samples were collected by retro-orbital bleeding, transferred into EDTA tubes (Thermo Fisher, #02-669-38CS), snap frozen, and stored at –80°C. Treated mice were anesthetized with ketamine/xylazine at 80/10 mg per kg, perfused transcardially with 10 ml PBS, and harvested for tumors, lungs, and blood on day 8, 4 hours after the final dose of erdafitinib or 28 hours after the final dose of anti–PD-1 for immunohistochemistry (IHC), flow cytometry, and T-cell receptor (TCR) sequencing analyses.

Lung tumor volumes were calculated from MRI images using the 3D Slicer software (http://www.slicer.org). Mice that did not survive the first MRI session after recruitment or those that died of causes other than lung carcinogenesis were excluded from the study, and only those that met the criteria were included for both efficacy and survival reads.

IHC

Mouse lungs were fixed in 10% buffered formalin overnight, transferred to 70% ethanol, and then embedded in paraffin. Formalin-fixed, paraffin-embedded (FFPE) sections (5 μm) were cut for hematoxylin and eosin (H&E) staining and also stained for IHC (Supplementary Table S1). Tumor regions were quantitated using Aperio Imaging System from images of tumor nodules stained for the indicated markers and averaged, with a minimum sample size of 5 animals per treatment group. Ten images were acquired for each mouse for analysis.

Patients with KRAS-mutant and FGFR-mutant NSCLC were identified through an institutional database of patients who had undergone genotyping as previously described (20, 21). The study was conducted in accordance with ethical standards of the Declaration of Helsinki. All 89 patients were consented to an institutional review board (IRB)–approved protocol allowing specimen collection and clinical data on a correlative...
science study. Inclusion criteria specified that the patients needed to have a diagnosis of a thoracic malignancy and be receiving their treatment at DFCI. Only diagnostic tumor tissue was collected; tumor samples were fixed in formalin and embedded in paraffin according to standard laboratory pathology practice, and stored at the department of pathology at the Brigham and Women's Hospital (Boston, MA). FFPE tissue sections (4 μm) were stained for both PD-L1 and CD3 (Supplementary Table S1), and stained slides were digitally scanned using an Aperio XT instrument at 20× magnification. The whole tumor section was scanned on Aperio. PD-L1 staining was visually scored by a pathologist with the generation of the H-score: the percentage of area stained multiplied by the weighted intensity. CD3 quantification was performed by PathAI company (https://www.pathai.com/) on Aperio-scanned images.

Primary tissue dissociation and flow cytometry

Collected lungs were also submerged in PBS containing 5 mMol/L EDTA. Lung tissue was placed in gentleMACS C Tube (Miltenyi, #130-056-334) and dissociated in collagenase type IV (Worthington, #1S0418G) and DNase I (Roche #10104159001)—containing Hank's Balanced Salt Solution (HBSS, Gibco, #24202117) plus 1× HEPES (Gibco, #15630080). Tissues were dissociated using the gentleMACS Dissociator (Miltenyi) under program "lung_02." Samples were then incubated at 37°C for 30 minutes with gentle mixing on a rotator followed by a second dissociation using program "imputor_01." The dissociated lung samples were passed through a 70-μm filter (Falcon) and rinsed with RPMI (Gibco, #72400) containing 10% FBS (HyClone, #SH300088.03). Dissociated cells were stained for viability with the Zombie Aqua Fixable Viability kit (BioLegend, #423102) according to the manufacturer's protocol. Fc receptors were blocked with TruStain FcX (BioLegend, #420301) on ice for 15 minutes, followed by staining for surface proteins (Supplementary Table S1). Cells were fixed and permeabilized using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience, #00-5523-01) for intracellular staining (Supplementary Table S1). AccuCheck Counting Beads (Life Technologies, #PCB100) were added to each sample for cell number quantification. Samples were analyzed on a BD LSRFortessa X-20 equipped with FACSDiva software, and further data analysis was performed with FlowJo software (Tree Star). Markers and gating strategy for flow cytometry are shown in Supplementary Table S2.

TCRβ immunosequencing

Collected tissues (approximately 250 μL blood, or 10 mg fragment or 100k cells from dissociated tumor-bearing lung) were snap frozen and stored at −80°C until analysis. Immunosequencing of the CDR3 regions of mouse TCRβ chains was performed using the immunoseq-Assay (Adaptive Biotechnologies). Genomic DNA was extracted from cell suspensions using the QIAasy Blood and Tissue Kit (Qiagen), from tissue using the QIAasympDNA Mini Kit (Qiagen), and from blood using the QIAasympDNA Midi Kit (Qiagen). DNA content was measured using the DropSense 96 Spectrophotometer. Extracted genomic DNA (up to 2.67 μg) was amplified in a bias-controlled multiplex PCR (Adaptive Biotechnologies), followed by high-throughput sequencing. Sequences were collapsed and filtered to identify and quantitate the absolute abundance of each unique TCRβ CDR3 region for further analysis as previously described (22–24). Data are available at https://clients.adaptivebiotech.com/pub/palakunthi-2019-cir.

Statistical analyses of TCRB immunosequencing data

Clonality was defined as 1- Peilou eveness (25) and was calculated on productive rearrangements by

$$1 + \sum_{i}^{N} p_i \log_2(p_i) / \log_2(N)$$

where p is the proportional abundance of rearrangement i, and N is the total number of rearrangements. Clonality values range from 0 to 1 and describe the shape of the frequency distribution: Clonality values approaching 0 indicate a very even distribution of frequencies, whereas values approaching 1 indicate an increasingly asymmetric distribution in which a few clones are present at high frequencies. Clonality between experimental groups was compared using a two-tailed Wilcoxon rank sum test. Correlations between T-cell fraction or clonality and tumor size data were assessed using Spearman rank correlation after running a Shapiro–Wilks test for normality. Clonal expansion was quantified by differential abundance analysis of clone frequencies between two samples from the same individual, which uses a binomial test with an FDR of 1% as previously described (26). Hierarchical clustering of clones by frequency was performed using a Ward linkage with Euclidean distances. Statistical analyses were performed in R version 3.3.

Cell culture, in vitro studies, and Western blotting

The KATO III cell line was maintained in IMDM supplemented with 20% FBS, and the H441 cell line was grown in RPMI supplemented with 10% FBS. All cell lines were from ATCC. KATO III cells were cultured in the presence of 40 ng/mL human FGF-2 (Miltenyibiotech, #130-093-839) and human IFNγ (Thermo Fisher, #RIFNG100) at a concentration of 5 ng/mL. For FACS analysis, a day after plating 25,000 cells per well in a 96-well plate, cells were treated with 0.01 to 500 nmol/L erdafitinib (or BGJ398 or AZD4547, Selleck Chemicals) for 24 hours, and then collected, pelleted, and resuspended in FACS Buffer (PBS + 2% FBS). Cells were stained with anti-human CD27 (B7-H1, PD-L1; BioLegend, #329707) and Zombie Violet Viability Kit (BioLegend, #423113) and analyzed by flow cytometry as described above.

For Western blotting, 750,000 cells were plated per well in a six-well plate and 24 hours later, were treated with 0.1 to 500 nmol/L erdafitinib (or rapamycin, trametinib, ruxolitinib, or SH4-54; Selleck Chemicals) for 1.5 hours. Cells were collected and lysed in RIPA lysis buffer (Boston BioProducts) with 0.5 M EDTA, 1 mMol/L dithiothreitol (DTT), 1 mMol/L phenylmethylsulfonyl fluoride (PMSF), and 1× Halt protease inhibitor cocktail (Thermo Fisher Scientific). Following rotation at 4°C for 20 minutes, lysates were cleaned by centrifugation (16,000 × g) for 15 minutes. Cleared lysates were quantified using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. For each lysate, 30 μg of total protein was separated by SDS-PAGE (Bio-Rad, #4561085) and transferred to nitrocellulose (Thermo Fisher, IB23002). After blocking for 1 hour in 5% w/v bovine serum albumin (BSA) in Tris-buffered saline (TBS; 0.1% Tween 20) buffer, membranes were
were stained for various proteins (Supplementary Table S1; antibodies diluted in 5% BSA in TBST at dilutions recommended by manufacturer) overnight at 4°C with gentle rocking. Blots were stained with HRP Radish Peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories) at 1/4,000 dilution in 5% BSA in TBST for 1 hour. Blots were incubated for 90 seconds in the SuperSignal West Pico or Femto Chemiluminescent Substrate Kit (Thermo Scientific, PC25923 or 34094, respectively), and staining was visualized using a FluorChem imaging system (ProteinSimple).

Analysis of FGFR2 mutation data across adenocarcinoma and squamous NSCLC
Mutation data were compiled from Genomics Evidence Neoplasia Information Exchange (GENIE; ref. 27; version 4.0) and The Cancer Genome Atlas (TCGA) (12). Lung adenocarcinomas (28) were selected for the duration of the experiment and were treated with increasing concentrations of erdafitinib (0.0000077, 0.000023, 0.000070, 0.000021, 0.000063, 0.000188, 0.000565, 0.01694, 0.051, 0.152, 0.457, 1.372, 4.115, 12.346, 37.037, 111.111, 333.333, and 1000 nmol/L). On days 2 and 6 after plating, cell viability was assessed by CellTiter-Glo (Promega). Reactions were run in triplicate.

Cytomegalovirus recall assays. In the cytomegalovirus (CMV) recall assays, 150,000 PBMCs isolated from healthy donors (Biological Specialty Corporation) plated 200,000 cells/well, were either unstimulated or stimulated with 1 ng/mL anti-CD3 antibodies (clone OKT3; Janssen) for the duration of the experiment and were treated with increasing concentrations of erdafitinib (0.0000077, 0.000023, 0.000070, 0.000021, 0.000063, 0.000188, 0.000565, 0.01694, 0.051, 0.152, 0.457, 1.372, 4.115, 12.346, 37.037, 111.111, 333.333, and 1000 nmol/L). On days 2 and 6 after plating, cell viability was assessed by CellTiter-Glo (Promega). Reactions were run in triplicate.

Results
Antitumor response and improved survival with erdafitinib and anti–PD-1 combination
The efficacy of the pan-FGFR inhibitor erdafitinib, both as a single agent and in combination with anti–PD-1, was evaluated in Lox-Stop-Lox-FGFR2<sup>K660N;p53mut</sup> (FKNP) mice, a fully immunocompetent genetically engineered mouse model (GEMM) of lung cancer driven by an inducible activating mutation in the kinase domain of FGFR2 (7). FKNP mice develop lung adenocarcinomas with a latency of approximately 35 weeks after intratracheal delivery into lungs of adenovirus-expressing Cre recombinase. Mutations in FGFR2, including in the kinase domain, have been reported in both squamous and adenocarcinoma NSCLC based on mutation data compiled from the GENIE and TCGA data sets (Supplementary Table S3), highlighting the clinical relevance of this model. FGFR-altered human cancers have been previously correlated with a non-T cell–inflamed TME (29). To determine if FKNP tumors shared features of FGFR-driven human tumors, we used flow cytometry to evaluate the TME (Supplementary Fig. S1). We observed a significant decrease in T and natural killer (NK) cells relative to normal lung (Supplementary Fig. S1A–S1C) and an increase in CD11c<sup>+</sup>CD11b<sup>+</sup> tumor-associated alveolar macrophages (TAM; ref. 30), regulatory T cells (Treg), and exhaustion marker–positive (PD-1, TIM-3, and LAG-3) T cells (Supplementary Fig. S1F and S1H–S1N). The fraction of PD-1<sup>+</sup>–positive tumor cells was increased relative to normal lung (Supplementary Fig. S1O). Together, these data suggest an immune-suppressive microenvironment in FKNP lung tumors.

MRI-confirmed lung tumor–bearing mice were randomized into four different treatment groups and treated for 4 weeks, followed by survival monitoring (Fig. 1A; Supplementary Fig. S2A). All treatments were well tolerated, with no significant loss in body weight during duration of treatment (Supplementary Fig. S2B). Treatment with anti–PD-1 alone did not reduce tumor burden at any time point after treatment. In contrast, partial or complete tumor regressions (average of 80% tumor growth inhibition [TGI]) were observed in all mice treated with erdafitinib monotherapy or in combination with anti–PD-1 at 2 to 4 weeks after treatment initiation and persisted until week 6, 2 weeks after treatment termination (Fig. 1B and C; P = 0.0001 at 2 and 4 weeks). No differences in TGI were observed between erdafitinib monotherapy and anti–PD-1 combination groups within the first 6 weeks of MRI monitoring (Fig. 1B and C). However, a significant survival advantage was observed in mice treated with the combination (19.7 weeks) compared with control (10.2 weeks; P < 0.0005) and erdafitinib (13.4 weeks; P < 0.004) groups (Fig. 1D).
In contrast to the FKNP model, a KRAS-driven lung cancer GEMM was insensitive to erdafitinib alone or in combination with anti–PD-1 (Supplementary Fig. S3A and S3B; Supplementary Table S4), suggesting that the observed effects were mediated through FGFR blockade in the FKNP model.

Erdafitinib alone or in combination with anti–PD-1 suppresses FGFR signaling

We next performed a separate short-term pharmacodynamic study in FKNP mice using the dosing and tissue collection schedule shown in Fig. 2A. As with the previous study (Fig. 1), over 90% of animals showed significant tumor regressions, as assessed by MRI (Fig. 2B and C; Supplementary Fig. S3A; Supplementary Table S5) after 1 week of treatment with erdafitinib alone or in combination with anti–PD-1. Under these treatment conditions, we profiled treatment-induced changes in signaling and the TME by IHC and flow cytometry. FGFRs signal through a key intracellular binding partner, FGFR substrate 2 alpha (FRS2α), that leads to MAPK and PI3K/AKT pathway activation (31, 32). A significant decrease in phospho-FRS2α (Y436) was observed 8 days after treatment with erdafitinib alone or in combination with anti–PD-1 (Fig. 2D–F), and decreased levels of phospho-S6 (S235/236) were seen in all treatment groups when compared with isotype control (Fig. 2E–G). These results indicated that constitutive FGFR activity and downstream signaling were suppressed by erdafitinib in FKNP mice, which correlated with antitumor activity.

Effects on immune cell infiltration and proliferation

To examine the basis for anti–PD-1 and erdafitinib combination on antitumor activity, we profiled treatment-induced changes in tumor and immune cell subsets (Fig. 3; Supplementary Fig. S4A–S4O). Consistent with the inhibition of FGFR signaling and tumor growth in erdafitinib-treated groups, a significant global decrease in Ki67+ proliferating cells was detected by IHC (Fig. 3A) compared with vehicle or anti–PD-1–treated animals. Evaluation by flow cytometry revealed that the proportion of proliferative epithelial cells (EpCAM+ Ki67+) was reduced by erdafitinib therapy alone or in combination with anti–PD-1 (Fig. 3C). We also examined treatment effects on different immune cells in the TME by both IHC and flow cytometry and observed a significant increase in infiltrating T cells in erdafitinib-treated groups (Fig. 3B and 3D–G; Supplementary Fig. S4P). A reduction in CD3+Ki67+ T cells was induced by erdafitinib (Fig. 3E; Supplementary Fig. S4K and S4L), which was paralleled by increases in central memory and effector CD4+ and CD8+ T cells (Fig. 3H and I; Supplementary Fig. S4N and S4O). Infiltration of CD8+ T cells was correlated...
Figure 2.
Inhibition of FGFR signaling in FKNP tumors. A, Pharmacodynamic study design. Pretreatment blood was collected a day before the start of the treatment. Treated mice were harvested for tumors and blood on day 8 for IHC, flow cytometry, and TCR sequencing analyses. Control \( n = 23 \), anti–PD-1 \( n = 20 \), erdafitinib \( n = 21 \), and combination \( n = 24 \). BID, every day; h, hours; QOD, every other day. B, Representative H&E sections of tumor-bearing lungs at day 8 of treatment for each group. Scale bar, 1 mm. C, Changes in percentage of tumor volume of individual mice in each treatment group after a week of treatment, quantified from MRI. ****, \( P < 0.0001 \), one-way ANOVA. Changes in expression of pFRS2 (D) and pS6 (E) in treatment groups were quantified using FFPE lung sections. *, \( P < 0.05 \); **, \( P < 0.01 \); ***, \( P < 0.0001 \), Welch t test. Representative IHC images for pFRS2 (F) and pS6 (G). Scale bar, 50 \( \mu \)m.
Figure 3.
Effects of erdafitinib and anti–PD-1 on T-cell infiltration and proliferation in FKNP tumors. Changes in immune cell infiltration and proliferation in FKNP tumor-bearing lungs at day 8 of treatment were analyzed. A and B, Representative IHC images (left) and quantified changes (right) by treatment are shown for Ki67⁺ (A) and CD3⁺ (B). Control n = 9, anti–PD-1 n = 5, erdafitinib n = 6, and combination n = 9. C-J, Flow cytometry analyses are shown for proliferative epithelial cells (EpCAM⁺Ki67⁺; C), T cells (CD3⁺; D), proliferative T cells (CD3⁺Ki67⁺; E), CD8⁺ cytotoxic T cells (CD8⁺; F), CD4⁺ helper T cells (CD4⁺; G), CD8⁺ central memory (CD8⁺CD62L⁺CD44⁺; H), and CD8⁺ effectors (CD8⁺CD62L⁻CD44⁻; I). Control n = 14, anti–PD-1 n = 13, erdafitinib n = 12, and combination n = 14. For A-J, *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001, Welch t test. J, Changes in infiltrating CD8⁺ and CD4⁺ cells are stratified by tumor response. Boxplots show minimum value, 25th percentile, median, 75th percentile, and maximum values. Responders: >30% tumor regression. *P = 0.0012; **P = 0.0001, two-tailed Wilcoxon rank sum test.
with antitumor responses (Fig. 3J). Changes in tumor-infiltrating lymphocytes (TIL) in the combination group were accompanied by a trend toward increased abundance of NK and B cells in the TME (Supplementary Fig. S4–S5). Overall, anti–PD-1 treatment enhanced proliferation of T and NK cells, such that anti–PD-1– and combination-treated tumors exhibited higher proportions of Ki67+ T cells and NK cells relative to the control and erdafitinib-treated groups, respectively, although the differences did not reach statistical significance (Fig. 3E; Supplementary Fig. S4K–S4M).

The fraction of infiltrating TAMs (CD11c+CD11b+) was decreased in combination-treated tumors compared with erdafitinib or anti–PD-1 monotherapy groups (Fig. 4A). Decreased proliferating TAMs were observed in both erdafitinib and combination groups (Fig. 4B). Erdafitinib-treated tumors exhibited a significant reduction in Tregs (Foxp3+CD25+CD4+) compared with the control group (Fig. 4C). Although erdafitinib treatment alone or in combination with anti–PD-1 did not significantly affect single exhaustion marker (PD-1, TIM-3, or LAG-3) expression on CD8+ or CD4+ T cells (Supplementary Fig. S5A and S5B), it did lead to a trend in the reduction of double exhaustion marker–positive T cells (PD-1+TIM-3–; Supplementary Fig. S5C and S5D) and a significant reduction in triple exhaustion marker–positive (PD-1+TIM-3+LAG3+) T cells (Fig. 4D and E), which are considered terminally exhausted T cells (33). Tumors that responded (>30% tumor regression) to erdafitinib monotherapy or in combination with anti–PD-1 therapy showed higher frequency of NK cells and lower frequency of TAMs (Fig. 4F). To determine whether erdafitinib directly affected immune cells, we tested treatment effects on the viability of human normal PBMCs (both resting or stimulated with anti-CD3; Supplementary Fig. S6A). T-cell viability remained constant with increasing concentrations of erdafitinib (Fig. 6B). To test the effect of gefitinib in the FKNP model, we treated naive mice with gefitinib (Supplementary Fig. S7A). Treatment with gefitinib did not significantly affect single exhaustion markers (PD-L1, TIM-3, or LAG-3) expression on CD8+ or CD4+ T cells (Supplementary Fig. S7B). In tumors, both T-cell fraction and clonality were increased after anti–PD-1 treatment alone. Clonality readout seemed to be inversely correlated with anti–PD-1 dose. T-cell fraction and clonality were increased after anti–PD-1 treatment compared with the control group (Fig. 6A). Treatment with erdafitinib resulted in decreased clonality, reflective of a T-cell population with a more balanced clone frequency distribution and, therefore, less clonal. The increased clonality observed in erdafitinib-treated tumors may reflect priming of immune responses through exposure of antigen-presenting cells (APC) to the tumor antigen repertoire as a result of tumor cell apoptosis directly induced by treatment. Combination with anti–PD-1 resulted in a significant increase in both T-cell fraction and clonality compared with erdafitinib treatment alone (Fig. 6A), consistent with anti–PD-1 treatment driving expansion of tumor-specific T-cell clones in the TME (36). A higher T-cell fraction (Fig. 6B) together with lower clonality (Fig. 6C) was observed in tumors that were responsive to erdafitinib and to a greater extent with the combination treatment (Fig. 6B). The clonality readout seemed to be inversely correlated with anti-tumor responses (Fig. 6C), suggesting that the increased clonality observed in the anti–PD-1 group was not productive.

Lung longitudinal analysis of the circulating T-cell repertoire revealed that clones that expanded in the blood were also present in the tumor (Fig. 6D), with the anti–PD-1 and anti–erdafitinib treatment groups trending toward higher numbers of these expanding clones. In the anti–PD-1– and combination-
treated tumors, clones that had low pretreatment frequency accounted for about half of the total number of expanded clones, suggesting that anti–PD-1 treatment results in the expansion of both existing and previously undetected clones. Erdafitinib predominantly caused expansion of clones that were undetectable (below detection limit) at baseline, consistent with the hypothesis that erdafitinib induces priming of previously undetected clones (Fig. 6E). Unsupervised clustering analysis of high-frequency tumor clones (at a frequency of 1% of the repertoire or greater) revealed that the T-cell repertoire of...
Figure 5.
Erdaftinib inhibits PD-L1 expression. Changes in PD-L1 expression in FKNP tumor-bearing lungs at day 8 of treatment were analyzed. Representative IHC images by treatment (A) and quantified changes by treatment (B). Scale bar, 200 μm. Flow cytometry showing the frequency of PD-L1^+EpCAM^+ cells (C) and PD-L1^+TAMs (D). PD-L1 expression was assessed in archived human lung cancer patient samples with FGFR alterations or KRAS mutations by IHC (E), with images representative of a wide range of PD-L1 expression (scale bar, 200 μm for FGFR and 400 μm for KRAS), and PD-L1 H-score (F), plotted vs. percentage of CD3 positivity per sample for patients with FGFR (n = 6) or KRAS (n = 83) alterations. Kato III cells (FGFR2-amplified human gastric cancer model) were treated with 0 to 500 nmol/L erdafitinib as indicated in the presence of IFNγ (5 ng/mL), and percentage decrease in PD-L1 expression relative to vehicle control-treated cells was assessed 24 hours later by flow cytometry (G) and FGFR signaling (H) evaluated 1.5 hours following treatment by Western blot. Samples cultured in the presence or absence of 5 ng/mL IFNγ included; tubulin probed as protein loading control. h, hours; NA, not applicable. **, P < 0.01; Welch t test.
individual mice was private, with few clones shared between mice within or across treatment groups (Supplementary Fig. S8).

**Discussion**

Targeted therapies can induce deep responses in patients with NSCLC by blocking actionable mutations, such as EGFR, that are essential for tumor cell growth and progression (37). In contrast, immunotherapy agents, such as those targeting the PD-1 pathway, have demonstrated clinical activity in patients by reactivating preexisting antitumor immune responses (38) but only benefit a subset of patients. Interestingly, driver pathway segments in NSCLC such as EGFR, ALK, and KRAS show limited benefit with immunotherapy, suggesting that these oncogenes...
induce changes in the TME, leading to escape from tumor immunosurveillance (39). As a result, significant efforts are ongoing to identify and develop combinations that could harness the nonoverlapping mechanisms of action of targeted agents and immunotherapy to broaden and increase the durability of clinical responses. Key to this concept is the ability of targeted therapies to induce immunogenic cell death that enhances tumor antigen presentation to T cells (40), whose functionality can be enhanced by the immune-activating potential of checkpoint inhibitors. In this study, we demonstrated that the combination of erdafitinib, a pan-FGFR small-molecule inhibitor (9), and PD-1 blockade led to inhibition of tumor growth and a survival advantage in FKNP mice, an FGFR2-driven autochthonous lung cancer GEMM (7). We provide evidence that the synergistic antitumor effect of this combination was dependent on erdafitinib-induced tumor cell killing, de novo priming, and enhancement of antitumor T-cell responses via PD-1 blockade.

Consistent with the previously described sensitivity of the FKNP model to FGFR inhibition (7), erdafitinib blocked FGFR signaling and exhibited potent antitumor efficacy. In contrast, this model was refractory to PD-1 blockade despite displaying high PD-L1 expression on both tumor and infiltrating immune cells. The combined inhibition of both FGFR and PD-1 led to similar initial tumor growth control compared with erdafitinib monotherapy but resulted in enhanced survival relative to the monotherapy-treated groups. Our data suggest that erdafitinib treatment leads to indirect enhancement of both adaptive and innate immunity in vivo, although it does not directly affect immune cell viability and responses in vitro. These effects were not observed in a KRASG12C-mutant GEMM that is insensitive to FGFR inhibition, indicating that the in vivo immune changes mediated by erdafitinib may be initiated as a consequence of tumor cell killing.

Erdafitinib treatment drove infiltration of both CD4+ helper and CD8+ effector T cells, while reducing the numbers of Tregs and terminally exhausted CD4+ and CD8+ T cells. This effect was dependent on tumor cell killing, because erdafitinib treatment in the nonresponsive KRASG12C-mutant model did not lead to a similar effect. Consistent with these data, it has been shown that direct killing of tumor cells with chemotherapy or targeted agents such as BRAF and MEK inhibitors causes immunogenic cell death and enhances immunogenicity by driving reexpansion of tumor antigens and T-cell infiltration, ultimately leading to increased sensitivity to checkpoint blockade (41, 42). Activation of the FGFR3 pathway is associated with non-T cell–inflamed tumors (29) resistant to checkpoint blockade, suggesting that inhibition of the FGFR pathway may be used as a means to elicit T-cell infiltration. Although erdafitinib induced changes in the TME consistent with an enhanced antitumor immune phenotype, this treatment alone did not result in survival benefit. In contrast, similar effects on infiltrating T cells in the anti–PD-1/erdafitinib combination group resulted in enhanced survival, suggesting that additional mechanisms drive more productive and durable immune responses with the combination in this model.

We hypothesized that the survival benefit observed in the combination-treated mice could result from anti–PD-1–mediated enhancement of antitumor T-cell responses primed by erdafitinib-induced cell killing. We proposed that these effects would be reflected in changes in the T-cell repertoire, which could be assayed by TCR sequencing of peripheral blood and tumors. In line with this hypothesis, treatment with erdafitinib resulted in a broader T-cell repertoire, consistent with priming of T-cell responses as a result of APC exposure to the diverse tumor antigen pool released following cell killing (43). Analysis of the peripheral T-cell repertoire supported this hypothesis by showing that erdafitinib-treated mice had higher numbers of previously undetected clones following treatment compared with baseline than other groups. Anti–PD-1 treatment resulted in increased clonality, reflecting a focusing of preexisting T-cell responses, as previously reported in both preclinical and clinical settings (36, 44). Our observation that groups treated with anti–PD-1, either as monotherapy or in combination with erdafitinib, had increased numbers of tumor-specific clones that were expanded in the periphery is consistent with results in the clinic, where tumors that exhibited a pathologic response to neoadjuvant anti–PD-1 treatment had higher frequency of T-cell clones that were shared between the tumor and periphery (45). The lack of monotherapy activity with anti–PD-1 in the FKNP model suggests that activation by anti–PD-1 of T-cell clones present at baseline in tumors may not be sufficient to drive productive antitumor responses. The combination of erdafitinib and anti–PD-1 led to an increase in T-cell clonality relative to erdafitinib monotherapy, suggestive of expansion of tumor-specific T-cell clones induced by erdafitinib. Therefore, our results support a model where erdafitinib primes the immune system by diversifying the T-cell repertoire, and PD-1 blockade drives clonal expansion and reinvigorates CD8+ TILs in the TME.

Erdafitinib and anti–PD-1 combination treatment induced unique changes in the TME in both lymphoid and myeloid populations, consistent with an antitumor phenotype. An orchestrated engagement of various immune cell populations, including decreased numbers of immunosuppressive TAMs, a trend toward increased DC activation, and NK and B cell infiltration, may be essential in the combination-treated mice to trigger deeper antitumor responses, especially in the context of cancers with limited tumor antigens, as represented by GEMM models (46). The shift in the microenvironment of combination-treated tumors toward a more productive inflammatory milieu likely supports and/or enhances antitumor immune responses (47). Limited studies to date have explored the effect of FGFR pathway modulation on the immune subsets mentioned above. For example, activation of FGFR1 has been shown to induce macrophage recruitment in tumors via CX3CL1 induction (48). Inhibition of FGFR has been reported to decrease myeloid-derived suppressor cells (MDSC) and enhance T-cell infiltration in 4T1 breast tumors, although these effects could be due to the additional targeting of CSFIR (49–51). Therefore, the mechanisms through which FGFR inhibition alone or in combination with PD-1 blockade alters specific immune subsets in the TME such as TAMs, DCs, and NK and B cells remain to be explored further.

Here, we showed that in human lung tumors, FGFR alterations were correlated with low T-cell infiltration independent of PD-L1 expression. Consistently, the TME in FKNP mice was characterized by high PD-L1 expression in both tumor and immune cells and by low T-cell infiltration. Treatment with erdafitinib led to PD-L1 downregulation in FGFR-expressing tumor cells (in vivo and in vitro), but not in macrophages. Our in vitro results show that erdafitinib reduced IFNγ-induced PD-L1 expression, suggesting that this could be the mechanism that leads to reduced PD-L1 in FKNP tumors. These data, together with the observation that erdafitinib had no effect on
Erdaftinib plus Anti–PD-1 Enhances Antitumor Responses


Disclosure of Potential Conflicts of Interest
E. Jazairy has ownership interest (including stock, patents, etc.) in Johnson & Johnson. C. Ferrante has ownership interest (including stock, patents, etc.) in Johnson & Johnson. J.M. English is Chief Scientific Officer at ILIOS Therapeutics and President and Head of Discovery, Immuno-Oncology, at EMD Serono. A.H. Beck has ownership interest (including stock, patents, etc.) in PathAI. J.A. Rylewski is Senior Computational Biologist at and has ownership interest (including stock, patents, etc.) in Adaptive Biotechnologies. C. Sanders has ownership interest (including stock, patents, etc.) in Adaptive Biotechnologies. K. Packman has ownership interest (including stock, patents, etc.) in Johnson & Johnson. P.A. Janne reports receiving commercial research grants from Astellas, AstraZeneca, Boehringer Ingelheim, Daiichi Sankyo, Takeda Oncology, and Eli Lilly, has ownership interest (including stock, patents, etc.) in Celecter Pharma, and LOXO Oncology, is a consultant/advisory board member for AstraZeneca, Boehringer Ingelheim, Vornono, Sfj Pharmaceuticals, Biocartis, ACEA Biosciences, Araxis, Ignity, Pfizer, Genentech/Roche, Merck, and Daiichi Sankyo, and Takeda Oncology, and has provided expert testimony for LabCorp. K.-K. Wong reports receiving a commercial research grant from Janssen Pharmaceuticals, MedImmune, Novartis, Pfizer, Merck, Takeda, and Mizati and has ownership interest (including stock, patents, etc.) in G1 Therapeutics. R.I. Verona has ownership interest (including stock, patents, etc.) in Johnson & Johnson. M.V. Lorenzi has ownership interest (including stock, patents, etc.) in Johnson & Johnson. No potential conflicts of interest were disclosed by the other authors.

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