INTRODUCTION

Lung cancer, in which about 80% of cases are classified as non–small cell lung cancer (NSCLC), is one of the leading causes of cancer-related mortality worldwide. Alterations in several oncogenic driver genes, including genes encoding epidermal growth factor receptor (EGFR) and anaplastic lymphoma kinase (ALK), have been reported in NSCLC. Molecular-targeted therapies directed against these driver gene alterations have been successfully developed, resulting in the improvement of patient prognosis (1, 2). The activating EGFR mutation is found in 50% of lung adenocarcinomas (LUADs) in East Asia, including Japan (3). Although patients with EGFR mutations initially respond to EGFR tyrosine kinase inhibitors, they usually become resistant to the therapy later. Thus, effective treatment strategies are urgently needed.

Recently, immune checkpoint blockade (ICB), including monoclonal antibodies (mAbs) against programmed cell death–1 (PD-1) and programmed cell death-ligand 1 (PD-L1), has demonstrated impressive antitumor effects in NSCLC, opening a new era in NSCLC treatment (4, 5). However, the efficacy is less than 50%, and development of treatments with increased efficacy is needed. Several approaches have been developed to augment the clinical efficacy of cancer immunotherapy, for example, combination with chemotherapy, anti–vascular EGF (VEGF) therapies, other ICBs, and regulatory T cell (Treg)–targeted therapies (6). Despite these promising results of ICB in NSCLC, a clinical efficacy of anti–PD-1/PD-L1 mAbs against EGFR-mutated NSCLC has been reported (4, 7, 8).

A retrospective study revealed that EGFR-mutated NSCLC has low expression rates of PD-L1, a predictive biomarker, and CD8+ tumor-infiltrating lymphocytes (TILs) (7). In contrast, other studies have shown that EGFR-mutated NSCLC cell lines have higher PD-L1 expression than EGFR wild-type NSCLC cell lines (9, 10).

Cancers with inherent genetic instability generate abnormal proteins, which have not been previously recognized by the immune system and become immunogenic antigens (neoantigens), thereby spontaneously triggering CD8+ T cell responses that contribute to elimination of the cancer cells from the hosts (11). To avoid immune cell attack, cancer cells establish immune-suppressive networks in the tumor microenvironment (TME), resulting in inflamed tumors characterized by concomitant infiltration of CD8+ T cells and immune-suppressive cells, such as Treg and myeloid-derived suppressor cells (MDSCs) (6). However, poorly immunogenic cancer cells that are selected during cancer development harbor low levels of neoantigens derived from gene alterations, leading to noninflamed tumors lacking both CD8+ T cells and immune-suppressive cells.

Cancers with oncogenic driver gene mutations, such as EGFR mutations, generally have a lower tumor mutation burden than cancers without these mutations, resulting in the development of a noninflamed TME (e.g., low levels of CD8+ T cells and immune-suppressive cells) (12, 13). In this study, we explored the immunological status of the TME in EGFR-mutated LUADs and identified an intriguing immunological status: high Treg infiltration without CD8+ T cell infiltration, which established a strong noninflamed TME. The intense noninflamed TME was attributed to the downstream signals of EGFR mutations that directly controlled T cell infiltration by changing the chemokine milieu in the TME. Therefore,
researchers should develop optimal cancer immunotherapy based on the immune phenotypes in the TME.

RESULTS
PD-L1 expression does not play an important role in preventing antitumor immune responses in EGFR-mutated LUADs

PD-L1 expression by tumor cells reduces effector T cell activity and promotes tumor progression (14). We first examined whether EGFR-mutated NSCLC cells had higher PD-L1 expression than EGFR wild-type NSCLC cells. Four cell lines (two EGFR-mutated and two EGFR wild-type NSCLC cell lines) were prepared from regular cultures without any stimulation, such as cytokines, and CD274 (encoding PD-L1) expression was examined. CD274 expression was higher in the EGFR-mutated NSCLC cell lines than in the EGFR wild-type NSCLC cell lines (fig. S1). To reflect the TME where tumor cells are exposed to interferon-γ (IFN-γ) produced by T cells and other immune cells, we added IFN-γ to the cultures of these NSCLC cell lines. CD274 expression was strongly elevated in both EGFR-mutated and EGFR wild-type NSCLC cell lines, resulting in comparable CD274 expression levels (fig. S1).

In addition, surgically resected tumor specimens from 19 patients with LUADs in which EGFR gene status had already been evaluated (6 EGFR-mutated and 13 EGFR wild-type LUADs) were subjected to RNA sequencing (RNA-seq). CD274 expression tended to be higher in EGFR wild-type LUAD than in EGFR-mutated LUAD, which was confirmed by immunohistochemistry (IHC), although the results were not significant (Fig. 1, A to C, and fig. S2). A dataset from The Cancer Genome Atlas (TCGA) also confirmed this trend (fig. S2). Thus, high PD-L1 expression in EGFR-mutated NSCLC cell lines, which has been shown in several previous reports (9, 10), did not reflect the TME in human NSCLCs and was not a major factor in raising antitumor immune responses in EGFR-mutated LUADs.

Immune-related gene expression and tumor mutation burden are decreased in EGFR-mutated LUADs

Nineteen LUAD samples subjected to RNA-seq were clustered on the basis of gene sets [CD4+ Treg, CD8+ T cells, macrophages, dendritic cells (DCs), major histocompatibility complex (MHC) class I, costimulatory antigen-presenting cells (APCs) and T cells, coinhibitory APCs and T cells, IFN response, and cytolytic activity] (12); 6 samples were inflamed (i.e., high-CD8+ T cell genes and high–cytolytic activity genes), and 13 samples were noninflamed (Fig. 1A). Of 13 noninflamed samples, 6 were EGFR-mutated LUADs, whereas all 6 inflamed samples were EGFR wild-type LUADs. EGFR-mutated LUADs showed substantially lower CD274, PDCD1 (encoding PD-1), CDRA, GZMA, and PRF1 expression than EGFR wild-type LUADs (Fig. 1B and fig. S2). There was no significant difference in smoking status, stage, or tumor size between the inflamed and noninflamed samples (Fig. 1A).

Next, whole-exome sequencing was performed with LUAD samples from which sufficient DNA samples were available. Both non-synonymous single-nucleotide variants and frameshift mutations, which can reflect the number of gene alteration-associated neoantigens and are associated with clinical efficacy of anti–PD-1 mAbs (11), were significantly higher in EGFR wild-type LUADs than in EGFR-mutated LUADs (Fig. 1D). TCGA data also confirmed the higher immune-related gene expression and tumor mutation burden in EGFR wild-type LUADs than mutated LUADs (fig. S2). These findings suggest that EGFR-mutated LUADs have a noninflamed TME with a low tumor mutation burden.

Increased prevalence of Treg in EGFR-mutated LUADs

In addition to gene assays of our 19 LUAD samples and TCGA data, flow cytometry and/or cytometry by time of flight (CyTOF) assays with TILs collected from 26 surgically resected LUADs (7 EGFR-mutated and 19 EGFR wild-type LUADs) were performed for detailed immune profiling of the TME. In EGFR-mutated LUADs, the frequency of CD8+ T cells was lower than that of the EGFR wild-type LUADs in CyTOF, consistent with the RNA-seq results. In addition, activated PD-1+CD8+ T cell and Gzmb+CD8+ T cell fractions were reduced in EGFR-mutated LUADs (Fig. 2A and fig. S3). FOXP3+CD4+ Treg, which are generally accompanied by effector T cells such as CD8+ T cells (15), were highly detected in EGFR-mutated LUADs (Fig. 2A).

To validate these data, we also investigated TILs with flow cytometry. Correct identification of Treg in humans is compromised because of the up-regulation of FOXP3 upon T cell receptor stimulation in conventional T cells (16). We have therefore proposed a classification of human Treg based on the expression levels of a naive marker CD45RA and FOXP3, and FOXP3+CD4+ T cells can be divided into three fractions: naive Treg (fraction I: nTreg, CD45RA+FOXP3lowCD4+); effector Treg (fraction II: eTreg, CD45RA-FOXP3highCD4+), with strong immune suppressive functions; and non-Treg (fraction III: CD45RA-FOXP3lowCD4+) without suppressive functions (Fig. 2B) (17–19). TIL analyses with flow cytometry confirmed that the frequency of CD8+ T cells tended to be lower in EGFR-mutated LUADs than in EGFR wild-type LUADs (Fig. 2B). The frequency of tumor-infiltrating eTreg and the eTreg/CD8+ T cell ratio were significantly higher in EGFR-mutated LUADs than in EGFR wild-type LUADs, corresponding to the data from CyTOF and IHC (Figs. 1C and 2, A and B). These findings suggest that Treg infiltrate into the TME despite the low levels of CD8+ effector T cells in EGFR-mutated LUADs. In contrast, only 2 of 19 patients with EGFR wild-type LUADs (AD no. 15 and no. 16) had a high eTreg/CD8+ T cell ratio (>0.2) (Fig. 2B), and FOXP3 expression in these patients was very high in accordance with the inflamed TME (Fig. 1A and fig. S2). Such patients seemed to have “inflammation-related acquired Treg” in the TME, and indeed, the FOXP3 gene, a representative Treg-related gene, was clustered into inflamed gene sets (Fig. 1A and fig. S4A) (15). There was no significant correlation between smoking status, stage, or tumor size and CD8+ T cell or eTreg infiltration (fig. S5).

In addition to Treg infiltration, tumor-associated macrophages (TAMs; CD68+CD163+CD206+ cells), MDCSs (CD33+CD11b+ cells), and DCs (CD11c+CD11b-HLA-DR+ cells) were analyzed with multiplex fluorescent IHC. In EGFR-mutated LUADs, the frequencies of TAMs, MDCs, and DCs tended to be slightly, but not significantly, higher than those of EGFR wild-type LUADs, which is consistent with the RNA-seq data (Fig. 1A and fig. S6).

Chemokine changes by EGFR signals are associated with the immune phenotypes in EGFR-mutated LUADs

To gain insight into the mechanism(s) for this immunological status of EGFR-mutated LUADs (high Treg infiltration despite low CD8+ effector T cell infiltration), we investigated the effect of EGFR signaling on CD8+ effector T cells and Treg infiltration with two EGFR-mutated cell lines (PC-9 and HCC827) and an EGFR wild-type cell line (H322) treated with erlotinib and EGF, respectively. Comprehensive

gene expression was analyzed with a microarray, and gene set enrichment analysis (GSEA) revealed that the gene signature of INTERFERON_GAMMA_RESPONSE, which is associated with chemokine production, was commonly enriched in the down-regulated state of the EGFR signal (fig. S7). Consistently, we found that gene ontology (GO) terms, cytokines, and chemokines, such as CCL5 and CXCL10, which reportedly recruit CD8+ T cells (20, 21), were downregulated by EGFR signaling (Fig. 3A). In addition, CCL22, which recruits Tregs (19, 22, 23), was elevated with activation of EGFR signaling (EGFR-mutated cell lines without erlotinib and EGFR wild-type cell line with EGF) (Fig. 3A). This elevation was abrogated by inhibition of EGFR signaling with an erlotinib in EGFR-mutated cell lines. The changes in mRNA and protein expression were observed with both erlotinib and third-generation EGFR tyrosine kinase inhibitor (osimertinib), as shown by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) and by enzyme-linked immunosorbent assay (ELISAs) (Fig. 3, B and C, and figs. S8 and S9).

EGFR signaling controls the transcription factors JNK/cJUN and IRF1 for the immune phenotype of EGFR-mutated LUADs

To further examine chemokine changes by EGFR signaling, we analyzed the transcriptional regulation of these chemokines. Because the JNK (cJun N-terminal kinase)/cJun pathway has been reported to increase CCL22 expression (24), JUN expression was examined. JUN expression was augmented along with CCL22 expression (Fig. 4, A and B). The increases in cJUN and phospho-cJUN were induced by phosho-JNK via EGFR signaling (Fig. 4C). In addition, JUN knockdown decreased CCL22 expression but not CXCL10 expression (Fig. 4D). A luciferase assay using CCL22 promoter regions...
also demonstrated that JUN knockdown decreased CCL22 luciferase activity (Fig. 4D), suggesting that EGFR signaling increases CCL22 expression via JNK/cJun activation.

To investigate the mechanism(s) of CCL5 and CXCL10 reduction, we examined a transcription factor(s) that showed comparable changes to CXCL10 in the microarray data. We found that interferon regulatory factor 1 (IRF1) expression was concurrently changed with CXCL10 expression (Fig. 4A) and was down-regulated by the activation of EGFR signaling (Fig. 4, A and B). The phosphatidylinositol 3-kinase/AKT pathway, which is downstream of the EGFR signal, has been reported to inhibit IRF1 expression (25, 26). Accordingly, pAKT was increased by the activation of the EGFR signal, consequently decreasing IRF1 (Fig. 4C). In addition, IRF1 knockdown resulted in the down-regulation of CXCL10, but not CCL22, at the mRNA level and in luciferase assays (Fig. 4E), indicating that the EGFR signaling decreased CXCL10 expression via IRF1 inhibition. We propose that the EGFR signaling plays an important role in driving high Treg infiltration despite low CD8+ effector T cell infiltration in EGFR-mutated LUADs via CCL22 up-regulation through JNK/cJun and CXCL10 down-regulation mediated by IRF1 (Fig. S4B).

A combination with erlotinib and anti–PD-1 mAb is a potential treatment strategy for EGFR-mutated LUADs

The functions of immune cells expressing EGFR might be directly modified by EGFR tyrosine kinase inhibitor (27, 28). To evaluate the direct effect of erlotinib on CD8+ T cells and Tregs, we
analyzed EGFR expression by immune cells in peripheral blood mononuclear cell (PBMC) and their sensitivity to erlotinib. Immune cells, including CD8+ T cells and Tregs, had limited expression of EGFR compared with lung cancer cell line PC-9 (fig. S10A). Accordingly, both CD8+ T cells and Tregs failed to respond to erlotinib treatment (fig. S10, B and C). In addition, phospho–Janus kinase 2 (pJAK2) and phospho–signal transducer and activator of transcription 5 (pSTAT5), which are downstream of the EGFR signal in immune cells (29), were not altered, and the expression levels of chemokines (CXCL10, CCL5, and CCL22) and transcription factors (IRF1 and JUN) were not changed (fig. S10, D and E). These findings indicate that the EGFR signal does not

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**Fig. 3.** CXCL10 recruiting CD8+ effector T cells is down-regulated and CCL22 recruiting Tregs is up-regulated by EGFR signal in EGFR-mutated LUADs. (A) Two EGFR-mutated cell lines (PC-9 and HCC827) and an EGFR wild-type cell line (H322) treated with erlotinib and EGF, respectively, were subjected to microarray analysis. GO terms, cytokines, and chemokines were examined. (B) CXCL10 and CCL22 expression levels in the EGFR-mutated cell lines (PC-9 and HCC827) treated with and without erlotinib and the EGFR wild-type cell line (H322) treated with and without EGF and erlotinib were evaluated by qRT-PCR. (C) The concentrations of CXCL10 and CCL22 in the cultured medium of the EGFR-mutated cell lines (PC-9 and HCC827) treated with and without erlotinib and the EGFR wild-type cell line (H322) treated with and without EGF and erlotinib were examined by ELISAs. Data are shown from three independent experiments.
directly influence on CD8\(^+\) T cells and T\(_{reg}\).

We next addressed whether EGFR signal inhibition altered the immunological status (high T\(_{reg}\) infiltration despite low CD8\(^+\) effector T cell infiltration) of EGFR-mutated LUADs and prevented tumor growth/progression. T\(_{reg}\) frequency in the TME of patients with EGFR-mutated LUADs who received erlotinib treatment was examined. T\(_{reg}\) infiltration was significantly reduced after erlotinib treatment (Fig. 5A), suggesting that a combination treatment with erlotinib and anti–PD-1 mAb could be possible. CXCL10 expression tended to be higher and CCL22 expression tended to be lower after erlotinib treatment than before erlotinib treatment in patients with EGFR-mutated LUADs, although there was no significant difference due to the small size of our patient cohort (fig. S11).

We then used human EGFR mutant (exon 19 deletion)–transfected mouse cell lines, a bulk cell line after transfection and two single
Fig. 5. The combination treatment with erlotinib and anti–PD-1 mAb effectively induces tumor growth inhibition in EGFR-mutated LUADs. (A) Left: Representative flow cytometry staining (CD45RA/FOXP3 for CD4+ T cells) of TILs. Right: A summary of patients with advanced LUAD treated with EGFR tyrosine kinase inhibitors (TKIs). TILs were collected before and after EGFR tyrosine kinase inhibitor treatment and subjected to flow cytometry. (B) Human EGFR wild-type or mutant (exon 19 deletion)–transfected MC-38 mouse cell line (MC-38wt or MC-38ex19del, respectively) was established, and human EGFR expression, phospho-EGFR, and exon 19-deleted EGFR were confirmed by Western blotting. (C) Mice were inoculated with MC-38wt or MC-38ex19del with and without erlotinib treatment, and tumor-infiltrating Treg and CD8+ T cells were analyzed.

Left: Representative flow cytometry staining of TILs. Right: A summary of the frequency of FOXP3+ CD4+ T cells and CD8+ T cells and ratio of FOXP3+/CD8+ T cells. (D) CXCL10, CCL22, CCL5, IRF1, and JUN expression levels in MC-38wt and MC-38ex19del with and without erlotinib were evaluated by qRT-PCR. Mice were inoculated with MC-38wt or MC-38ex19del with and without erlotinib treatment. Tumors were collected on day 8, and CXCL10, CCL22, CCL5, IRF1, and JUN expression was analyzed by qRT-PCR. (E) Tumor growth and the survival curve are shown. Representative data from two independent experiments are shown. (F) Human EGFR wild-type or mutant (exon 19 deletion)–transfected LL/2-OVA mouse cell line (LL/2-OVAwt or LL/2-OVAex19del, respectively) was established, and human EGFR expression, phospho-EGFR, and exon 19–deleted EGFR were confirmed by Western blotting. (G) Mice were intravenously administered with LL/2-OVAex19del and treated with and without erlotinib, anti–PD-1 mAb or a combination (erlotinib + anti–PD-1 mAb). A summary of lung weights with tumors is shown. Representative data are shown from two independent experiments.

clones of MC-38ex19del, to examine the in vivo antitumor activity (Fig. 5B and figs. S12A and S13). Compared with those of EGFR wild-type transfected cell line (MC-38wt)–derived tumors, higher and lower frequencies of Treg and CD8+ T cells, respectively, were observed in the TME of MC-38ex19del–derived tumors. The high Treg and low CD8+ T cell infiltration in the TME was totally abrogated by erlotinib treatment (Fig. 5C). In addition, consistent changes in chemokines and transcription factors were observed:

CXCL10, CCL5, and IRF1 were down-regulated in MC-38ex19del-derived tumors and were increased by erlotinib treatment. CCL22 and JUN were up-regulated in MC-38ex19del-derived tumors and were reduced by erlotinib treatment (Fig. 5D). Furthermore, when CXCL10 was blocked with an antibody, the elevation of CD8+ T cells in the TME induced by erlotinib treatment was abrogated (Fig. S14A), and no synergistic effect of erlotinib and anti-CCL22 mAb on Tregs in the TME was observed (fig. S14B). Consequently, the combination of erlotinib and anti–PD-1 mAb significantly inhibited tumor growth compared with the control or either single treatment using a bulk cell line after transfection and two single clones of MC-38ex19del (Fig. 5E and fig. S12B). In contrast, no synergistic effect of erlotinib and anti–PD-1 mAbs was observed in the control cell lines (MC-38mock and MC-38wt) (Fig. 5E). Moreover, this combination treatment exhibited a superior antitumor effect on orthotopic EGFR exon 19 deletion–transfected LL/2-OVA (LL/2-OVAex19del; a bulk cell line after transfection) than either treatment alone (Fig. 5, F and G). Additional Treg depletion with anti-CD25 mAb failed to show any synergistic or additive antitumor effects (fig. S15). Our results suggest that combination treatment with an EGFR tyrosine kinase inhibitor such as erlotinib and anti–PD-1 can be a promising strategy for the treatment of EGFR-mutated LUADs.

DISCUSSION

Cancers are immunologically divided into two major types, inflamed and noninflamed tumors. Tregs have been thought to be recruited by inflammation into the TME (“inflammation-related acquired Treg”) (fig. S4) (15), as was observed in EGFR wild-type LUADs. In this study, we identified an intriguing immunological status in EGFR-mutated LUADs: high Treg infiltration despite the noninflamed TME. We then proposed a concept of immune suppression, particularly by Tregs in the TME, “tumor-related innate Tregs” (fig. S4). The dependence of tumor growth and/or survival on driver gene alterations such as EGFR mutations or ALK rearrangements in NSCLC is known as oncogenic driver addiction. Patients with such oncogenic driver gene alterations respond to molecular-targeted therapies (1, 2), indicating an important role of such oncogenic driver gene alterations in cell growth or survival. In addition, we found that these gene alterations play another crucial role in immune responses through development of an immune-suppressive environment in the TME of EGFR-mutated LUADs. Together, driver genes contribute to not only cell growth and/or survival but also immune escape from antitumor immunity.

Several previous studies have demonstrated that EGFR-mutated NSCLC cell lines have higher expression of PD-L1, one of the predictive biomarkers of PD-1/PD-L1 blockade therapies (5), than EGFR wild-type NSCLC cell lines (9, 10). PD-L1 expression is induced by two different mechanisms: genetic alterations (i.e., amplification, fusion, and 3’ untranslated region disruption) (innate expression) and induction by inflammation (such as acquired IFN-γ expression) (14). We revealed that whereas EGFR-mutated NSCLC cell lines cultured with regular medium without any stimulation, such as cytokines, exhibited slightly higher CD274 (encoding PD-L1) expression than EGFR wild-type NSCLC cell lines, CD274 expression was strongly enhanced by IFN-γ, showing a strong elevation of CD274 expression in both EGFR-mutated and EGFR wild-type NSCLC cell lines regardless of their original expression. The extent of CD274 elevation induced by IFN-γ was significantly higher in EGFR wild-type NSCLC cell lines than in EGFR-mutated NSCLC cell lines. CD274 expression is primarily regulated by IFN-γ via IRF1 (30), and our study revealed that EGFR signaling negatively regulated IRF1. Therefore, IFI19 is suppressed by EGFR signaling in EGFR-mutated LUADs, resulting in a low increase in CD274 expression by IFN-γ. RNA-seq and IHC exhibited higher PD-L1 expression in EGFR wild-type LUADs than in EGFR-mutated LUADs, and PD-1 blockade resulted in poor clinical responses in EGFR-mutated LUADs (4, 7, 8).

The clinical responses of anti–PD-1/PD-L1 mAbs against EGFR-mutated LUADs were unfavorable because of the low tumor mutation burden, low PD-L1 expression, and the noninflamed TME (7, 8). Tumor mutation burden can be reflected in the number of neoantigens derived from gene alterations, which induce a strong immune response as nonspecific antigens, leading to an inflamed TME. Thus, tumor mutation burden is reportedly associated with the clinical efficacy of anti–PD-1/PD-L1 mAbs (11). In our analyses, because tumor mutation burden was low in EGFR-mutated LUADs, immune-related gene expression was low in EGFR-mutated LUADs. Treg infiltration, which was frequently accompanied by CD8+ T cell infiltration in the inflamed TME (15), in EGFR-mutated cancers tended to be higher or comparable with that in EGFR wild-type cancers, although CD8+ T cell infiltration was limited in EGFR-mutated cancers. Therefore, the TME (high Treg infiltration without CD8+ T cell infiltration) was not developed solely because of low tumor mutation burden; rather, the immunological effects of EGFR mutations must be strongly involved via prevention of the recruitment of effector CD8+ T cells by down-regulation of CXCL10 through IRF1 and promotion of Treg infiltration by up-regulation of CCL22 through JNK/cJUN. Considering that Tregs hamper the development of effective antitumor immunity in tumor-bearing hosts (19), the immunological status in the TME induced by EGFR mutations can be associated with resistance to cancer immunotherapy, as observed in our in vivo study, suggesting that combination treatment of anti–PD-1 mAb and EGFR signal inhibitors should augment the antitumor efficacy. In patients with advanced EGFR-mutated LUADs, tumor-infiltrating eTreg frequency was significantly lower after erlotinib treatment. Although several clinical trials of EGFR tyrosine kinase inhibitors in combination with anti–PD-1 mAb have been performed, the high incidence of treatment-related adverse effects limited successful clinical application. In contrast, a recent phase 3 trial demonstrated that anti–PD-L1 antibody combined with bevacizumab, an anti-VEGF therapy, exhibits clinical efficacy against EGFR-mutated NSCLC (31). Antiangiogenic drugs reportedly reduced Treg (32), which can partially explain the superior clinical efficacy of the combination therapy against EGFR-mutated NSCLC. However, the tumor mutation burden is lower in EGFR-mutated NSCLC than in EGFR wild-type NSCLC, indicating smaller numbers of neoantigens. In addition to regulating EGFR signaling, combination strategies that elicit CD8+ T cells against cancer antigens, although the number is limited, may provide notably favorable clinical efficacy in EGFR-mutated NSCLC.

Chemokine production is controlled by multiple components, such as tumor cells and immune cells (33). Consistent with this finding, the basal level of CCL22 production in the EGFR wild-type H322 cell line was higher than in other EGFR-mutated cell lines. Inflammatory signals provided in the TME may imprint CCL22 production in the H322 cell line. Therefore, although CCL22 expression is regulated by the JNK/cJUN signal, which is downstream of the EGFR signal in our data, many other signals may regulate
CCL22 expression (34). Thus, tumor cell line information may not
directly influence the TME. In MC-38 tumor model in which we can
directly examine the role of EGFR signaling in CCL22 expression,
MC-38 with an EGFR mutation increased CCL22 expression
compared with wild-type MC-38, resulting in enhanced Treg
infiltration in the TME. These findings indicated the critical roles of
chemokines derived from EGFR-mutated cancers in both Treg and
CD8+ T cell infiltration in the TME. The other chemokine that
showed elevation with EGFR signal activation was CXCL8, which
reportedly mainly recruited neutrophils (35). In contrast, CCL21 (a
ligand of CCR7), which mainly promoted the chemotaxis of natural
killer T cells and naive T cells (36), respectively, were down-
regulated by the EGFR signals, consistent with the noninflamed TME in
EGFR-mutated cancer (fig. S16). However, consistent tendencies
were not observed in the RNA-seq data.

Whereas EGFR expression by immune cells, including Tregs, has
been detected (27, 28), we found limited expression of EGFR in Tregs
and CD8+ T cells. Accordingly, erlotinib treatment did not influence
the viability or function, including chemokines, of these cells. In
addition, synergistic antitumor effects by the combination of erlotinib
and anti–PD-1 mAb in EGFR wild-type cancers were not observed
when compared with that of anti–PD-1 mAb alone. As EGFR
expression by Tregs, was reported in inflammatory conditions (27), one
plausible explanation is that immune cells, particularly Tregs, use vari-
dous different signals for molecular expression depending on each
condition, such as cancers and inflammation. In EGFR-mutated
cancers, the noninflamed immune-suppressive TME (high Tregs
and low CD8+ T cells) may reduce the expression of EGFR by
immune cells such as Tregs.

In conclusion, we found an intriguing immunological status in
the TME of EGFR-mutated LUADs: high Treg infiltration despite the
noninflamed TME. Tregs are primarily recruited via signals from
tumor cells (“tumor-related innate Tregs”), which are induced by
driver gene alterations such as EGFR mutations and related to
resistance to cancer immunotherapies. Driver gene alterations rep-
resented by EGFR mutations therefore play an important role in
cell growth and/or survival and the development of immune escape
mechaneries, warranting further tests in cancer immunotherapies
combined with molecular-targeted therapies against cancers with
driver gene alterations.

MATERIALS AND METHODS
Patients and samples
Peripheral blood and tumor tissues were obtained from patients
with LUADs who underwent surgery at Osaka University Hospital
and National Cancer Center Hospital East from 2014 to 2015 and
advanced LUAD patients harboring EGFR mutations who received
EGFR tyrosine kinase inhibitors, such as gefitinib, erlotinib, and
afatinib, treatment at National Cancer Center Hospital East from
2015 to 2016 (summarized in tables S1 and S2, respectively). PBMCs
were isolated by density gradient centrifugation with Ficoll-Paque
(GE Healthcare, Little Chalfont, UK). For collection of TILs, tumor
tissues were minced and treated with gentleMACS Dissociator
(Miltenyi Biotec, Bergisch Gladbach, Germany) as described previ-
ously (18). PBMCs from healthy individuals were purchased from
Cellular Technology Limited (Cleveland, OH). PBMCs were cultured
in RPMI 1640 supplemented with 10% AB serum. PBMCs were
cultured with CD3/CD28 Dynabeads (Thermo Fisher Scientific,
Waltham, MA) according to the manufacturer’s instructions for
3 days. All donors provided written informed consent before sam-
pling, according to the Declaration of Helsinki. This study was per-
formed in a blinded and nonrandomized manner and was approved
by Osaka University Hospital Ethics Committee and National Cancer
Center Ethics Committee.

IHC for PD-L1, CD8, and FOXP3
The antibodies used in IHC are summarized in table S3. Surgically
resected samples were formalin fixed, paraffin embedded, and sec-
tioned onto slides for IHC. The slides were deparaffinized with
xylene, rehydrated, and antigen-retrieved in a microwave oven for
20 min. After the inhibition of endogenous peroxidase activity,
individual slides were then incubated overnight at 4°C with a mouse
anti-human CD8 mAb, a rabbit anti-human FOXP3 mAb, and a
rabbit anti-human PD-L1 mAb. The slides were then incubated with
EnVision reagent (Dako, Glostrup, Denmark), and a color reaction
was developed in 2% 3,3-diaminobenzidine in 50 mM tris buffer
(pH 7.6) containing 0.3% hydrogen peroxidase. Last, these sections
were counterstained with Mayer hematoxylin. PD-L1 positivity was
evaluated in the tumor cells. CD8 and FOXP3 staining was quantified
in five high-power microscopic fields (×400; 0.0625 mm2), and the
mean values were calculated. Two pathological researchers (E. Sugiyama
and G.I.) independently evaluated the stained slides.

Multiplex immunofluorescence staining
The antibodies used in multiplex immunofluorescence staining are
also described in table S3. Surgically resected samples were formalin
fixed, paraffin embedded, and sectioned onto slides. The slides were
deparaffinized with xylene, rehydrated, and antigen-retrieved in a
microwave oven for 40 min. After the inhibition of endogenous
peroxidase activity, individual slides were then incubated for 1 hour
at room temperature with a rabbit anti-human CD33 mAb, a rabbit
anti-human CD11b mAb, a rabbit anti-human CD11c mAb, a mouse
anti-human CD68 mAb, a mouse anti-human CD163 mAb, a
mouse anti-human CD206 mAb, and a rabbit anti-human HLA-DR mAb.
Anti-rabbit/mouse polymeric horseradish peroxidase (System–HRP–
labeled polymer anti-rabbit, EnVision, Dako) was applied as the
secondary label for 20 min. Signals from the antibody complexes
were visualized with their corresponding Opal Fluorophore Reagents
(PerkinElmer, Waltham, MA) after incubation of the slides for
10 min. Slides were air-dried, mounted with ProLong Diamond
Antifade mounting medium (Thermo Fisher Scientific), and stored
in a light-proof box at 4°C before imaging. Multiplexed fluorescent
labeled images of one to five randomly selected fields (669 μm by
500 μm) were captured with an automated imaging system (Vectra 3,
PerkinElmer). Cell counts were determined manually for each image.

CyTOF analysis
CyTOF staining and analysis were performed as described (37).
The antibodies used in the CyTOF analyses are summarized in table
S4. Cells were subjected to staining after they were washed with
phosphate-buffered saline (PBS) supplemented with 2% fetal calf
serum (FCS; washing solution) and then with PBS to reduce the pro-
tein concentration in the medium, which interferes with the sub-
sequent dead cell staining by cisplatin. The cells were incubated
in 5 μM Cell-ID Cisplatin solution (Fluidigm catalog no. 201064,
South San Francisco, CA) in PBS, washed using washing solution,
and stained with a mixture of surface antibodies. After the cells were


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washed, they were fixed and permeabilized using Foxp3/Transcription Factor Staining Buffer Set (Thermo Fisher Scientific) according to the manufacturer’s instructions. The fixed and permeabilized cells were then stained with the intracellular antibodies. After the cells were washed twice, they were fixed in 125 mM MaxPar Intercalator-Ir (Fluidigm) diluted in 2% paraformaldehyde PBS solution at 4°C. The cells were then washed once with washing solution and twice with MaxPar water (Fluidigm catalog no. 201069), distilled water with minimal heavy element contamination, to reduce the background level. The cells suspended in MaxPar water supplemented with 10% EQ Four Element Calibration Beads (Fluidigm) were applied to the Helios instrument (Fluidigm), and data were acquired at a speed below 300 events/s.

Flow cytometry analysis
Flow cytometry staining and analysis were performed as described (37). The antibodies used in the flow cytometry analyses are summarized in table S5. Cells were washed using washing solution and subjected to staining with surface antibodies. Intracellular staining of FOXP3, pJAK2, and pSTAT5 was performed with anti-Foxp3 mAb, anti-pJAK2 mAb, and anti-pSTAT5 mAb and the Foxp3/Transcription Factor Staining Buffer Set (Thermo Fisher Scientific) according to the manufacturer’s instructions. After the cells were washed, they were analyzed with an LSRFortessa or FACSymphony (BD Biosciences, Franklin Lakes, NJ) and FlowJo software (TreeStar, Ashland, OR). The staining antibodies were diluted according to the manufacturer’s instructions.

RNA sequencing
After quality assessment with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA), polyadenylated RNA libraries were generated using a TruSeq Stranded mRNA Sample Prep Kit (Illumina, San Diego, CA) on an Agilent XT-Auto System (Agilent Technologies) and sequenced with a HiSeq SBS Kit v4-HS (Illumina) on HiSeq2500 (Illumina). Sequence data were evaluated with GeneData Expressionist for Genomic Profiling (version 9.1.4a). Read mapping was performed with Hg19 as the reference genome and TopHat (version 2.0.14), followed by transcriptome reconstruction and expression quantification into fragments per kilobase of transcript model per million (FPKM).

Whole-exome sequencing and mutational analysis
DNA libraries were established with a SureSelect XT Human All Exon system (Agilent Technologies) and sequenced with a HiSeq SBS Kit v4-HS (Illumina) on a HiSeq2500 system (Illumina) to generate paired-end reads (2 × 100 base pairs). Sequence alignment and mutation calling were performed using the Genome pipeline (https://github.com/Genomon-Project/), as described previously (38). Candidate mutations were detected by the Empirical Bayesian Mutation Calling (EBCall) algorithm, and those with (i) a P value <10⁻⁴, (ii) >4 variant reads in tumor samples, and (iii) a variant allele frequency (VAF) value in tumor samples of >0.025 were adopted. These candidate mutations were further filtered by excluding (i) synonymous single-nucleotide variants; (ii) known variants listed in the 1000 Genomes Project (October 2014 release), National Center for Biotechnology Information (NCBI) dbSNP build 131, National Heart, Lung, and Blood Institute Exome Sequencing Project 6500, Human Genome Variation Database, or our in-house single-nucleotide polymorphism database; and (iii) variants present only in unidirectional reads.

Gene expression data analysis
In addition to our RNA-seq dataset, a TCGA dataset of LUADs was also analyzed. The EGFR gene status and mutation burden for 230 LUADs were evaluated on the basis of previously published reports, and gene expression profiles and nonsynonymous mutations of these samples were extracted from the TCGA data portal (https://tcga-data.nci.nih.gov/tcga/tcgaHome2.jsp). For clustering, we used Cluster 3 for CD4⁺ Treg, CD8⁺ T cell, macrophage, DC, MHC class I cell, costimulatory APC and T cell, coinhibitory APC and T cell, type I IFN response, type II IFN response, and cytolytic activity gene sets as previously reported (12).

Gene set enrichment analysis
GSEA was carried out to analyze the differences between two groups: activated EGFR signaling and inhibited EGFR signaling in three lung cancer cell lines (PC-9, HCC827, and H322). The gene sets were adopted from The Molecular Signatures Database. The phenotype label was EGFR activation score versus EGFR inhibition score.

Gene expression analysis using the nCounter platform
For RNA purification, 10-μm formalin-fixed, paraffin-embedded slides were used for each tumor specimen. RNA was extracted using the RecoverAll Total Nucleic Acid Isolation Kit (Thermo Fisher Scientific). A minimum of 100 ng of total RNA was used to measure chemokine expression. Gene expression analyses were performed using the Human PanCancer IO 360 Panel and nCounter Low RNA Input Kit (NanoString Technologies, Seattle, WA). Data were normalized by nSolver analysis software.

Cell line and reagents
A549, H322, and HCC827 cells (human NSCLC cell lines) were obtained from American Type Culture Collection (ATCC; Manassas, VA) (ATCC catalog no. CRL-7909, catalog no. CRL-5806, and catalog no. CRL-2868, respectively), and PC-9 cell line (human NSCLC cell line) was obtained from the European Collection of Authenticated Cell Cultures (ECACC) (Salisbury, UK) (ECACC catalog no. 90071810). MC-38 cell line (mouse colon cancer cell line) was obtained from Kerafas (Boston, MA) (catalog no. EH204), and LL/2 cell line (mouse lung cancer cell line) was obtained from ATCC (ATCC catalog no. CRL-1642). All human cell lines were authenticated using a short tandem repeat DNA method. The A549 and LL/2 cell lines were maintained in Dulbecco’s modified Eagle’s medium (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) supplemented with 10% FCS. The H322, HCC827, and MC-38 cell lines were maintained in RPMI medium (FUJIFILM Wako Pure Chemical Corporation) supplemented with 10% FCS. The human EGFR (wild type or exon 19 deletion)–overexpressing MC-38 and LL/2-OVA cell lines were established retrovirally using a pBabe-puro vector (Addgene, Cambridge, MA) (named MC-38wt, MC-38ex19del, LL/2-OVAwt, and LL/2-OVAex19del cell lines). Erlotinib was obtained from Cayman Chemical Company (Ann Arbor, MI), osimertinib was purchased from Selleck (Houston, TX), anti-mouse PD-1 mAb was provided by Ono Pharmaceutical (Osaka, Japan), anti-mouse CXCL10 mAb (clone 134013, R&D Systems, catalog no. MAB466–100) and anti-mouse CCL22 mAb (clone 158132, R&D Systems, catalog no. AF439) were obtained from R&D Systems (Minneapolis, MN), and anti-mouse CD25 (clone PC6G1, Biolegend, catalog no. 102040) was purchased from BioLegend (San Diego, CA). IFN-γ and EGF were purchased from PeproTech (Rocky Hill, NJ).
**qRT-PCR and microarray analyses**

Total RNA was reverse-transcribed to complementary DNA using a SuperScript VILO Master Mix according to the manufacturer’s instructions (Thermo Fisher Scientific), and real-time PCR was performed with PowerUp SYBR (Thermo Fisher Scientific). The glyceraldehyde-3-phosphate dehydrogenase gene was used as an endogenous control, and the primers used are summarized in table S6. Microarray analysis was performed using the Clarion S array according to the manufacturer’s instructions (Thermo Fisher Scientific).

**Enzyme-linked immunosorbent assay**

The concentrations of CXCL10 and CCL22 were examined with a specific sandwich ELISA according to the manufacturer’s instructions (R&D Systems).

**Western blotting**

Subconfluent cells were washed with PBS and harvested with mammalian protein extraction reagent (M-PER) (Thermo Fisher Scientific). Whole-cell extracts were separated with SDS–polyacrylamide gel electrophoresis and were blotted onto a polyvinylidene fluoride membrane. After the membrane was blocked, it was probed with the primary antibody. After the membrane was rinsed twice with tris-buffered saline buffer, it was incubated with a horseradish peroxidase–conjugated secondary antibody and washed, followed by visualization using an enhanced chemiluminescence detection system and a LAS–4000 (GE Healthcare). The antibodies used in Western blot analyses are summarized in table S7.

**Small interfering RNA**

Cells were transfected with a small interfering RNA (siRNA) for JUN or IRF1 and a nonspecific target (control) using RNAiMAX (Thermo Fisher Scientific). ON-TARGETplus Human JUN siRNA SMART pool (Dharmacon catalog no. L-003268-00-0005, Lafayette, CO), ON-TARGETplus Human IRF1 siRNA SMART pool (Dharmacon catalog no. L-011704-00-0005), and ON-TARGETplus Non-targeting Pool (Dharmacon catalog no. D-001810-1005) were used.

**Luciferase assay**

A pNL2.1 vector (Promega catalog no. N1061, Madison, WI) containing the CXCL10 or CCL22 promoter region upstream of the luciferase gene was generated. Luciferase activity was determined using the Luciferase Assay System (Promega). The results are reported as the fold induction compared with the control group.

**In vivo animal model**

C57BL/6 mice (6-week-old females; CLEA Japan, Tokyo, Japan) were used for the in vivo studies. Animal care and experiments were conducted according to the guidelines established by the animal committee of the National Cancer Center after approval of the Ethics Review Committee for Animal Experimentation of the National Cancer Center. A suspension of 1 × 10⁶ transfected cells (in 100 μl of PBS) was subcutaneously (MC-38) or intravenously (LL/2-OVA) administered, and treatment was started after 1 week, when tumors in each group reached an average volume of about 500 mm³. In some groups, anti–PD-1 mAb (200 μg per body intraperitoneally) was administered at 1-week intervals with or without oral daily erlotinib (30 mg/kg) for 3 weeks. The tumor volume was assessed twice a week as the length × width² × 0.5. For blocking of CXCL10 and CCL22, 50 μg of anti-mouse CXCL10 mAb and 20 μg of anti-mouse CCL22 mAb, respectively, were administered intraperitoneally on days 4 and 7 after tumor implantation. For depletion of Treg, 200 μg of anti-mouse CD25 mAb was administered intraperitoneally on day 7 after tumor implantation. TIL analysis was performed on day 10.

**WST1 assay**

The WST1 assay was performed according to the manufacturer’s instructions (Roche, Basel, CH) to evaluate the sensitivities to erlotinib and cell proliferation. After each cell line was seeded in a 96-well plate, the cells were incubated with erlotinib for 48 hours. Then, WST1 reagent (10% of medium) was added, and absorbance was analyzed by a microplate reader at 450 and 690 nm. The proliferation of the transfected MC-38 cell lines was analyzed in the same way without erlotinib 0, 24, and 48 hours after seeding.

**Statistical analysis**

Continuous variables were analyzed with Welch’s or paired t tests. Survival curves were estimated with the Kaplan-Meier method and compared with the log-rank test. The statistical analyses were performed with Prism version 7 software (GraphPad Software Inc., La Jolla, CA). A P value of less than 0.05 was considered statistically significant.


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Blockade of EGFR improves responsiveness to PD-1 blockade in EGFR-mutated non-small cell lung cancer

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Giving anti-PD-1 a boost

Anti-PD-1 therapy is ineffective in the context of EGFR-mutated lung adenocarcinomas (LUADs). Here, Sugiyama et al. find that these tumors have an immunosuppressive tumor microenvironment characterized by increased infiltration of regulatory T cells. Using a mouse model, they demonstrate that a clinically available EGFR inhibitor, erlotinib, can be used to improve responsiveness to anti-PD-1 therapy in EGFR-mutated LUADs. Because both anti-PD-1 antibodies and erlotinib are already being used in the clinic, this preclinical proof-of-concept study should serve as a basis for clinical studies to examine whether erlotinib enhances responsiveness of EGFR-mutated lung adenocarcinomas to PD-1-centric therapies.