A High-Throughput Immune-Oncology Screen Identifies EGFR Inhibitors as Potent Enhancers of Antigen-Specific Cytotoxic T-lymphocyte Tumor Cell Killing

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Abstract

We developed a screening assay in which luciferized ID8 expressing OVA was cocultured with transgenic CD8+ T cells specifically recognizing the model antigen in an H-2b–restrict- ed manner. The assay was screened with a small-molecule library to identify compounds that inhibit or enhance T cell–mediated killing of tumor cells. Erlo tinib, an EGFR inhibitor, was the top compound that enhanced T-cell killing of tumor cells. Subsequent experiments with erlotinib and additional EGFR inhibitors validated the screen results. EGFR inhibitors increased both basal and IFNγ-induced MHC class-I presentation, which enhanced recognition and lysis of tumor cell targets by CD8+ cytotoxic T lymphocytes. The ID8 cell line was also transduced to constitutively express Cas9, and a pooled CRISPR screen, utilizing the same target tumor cell/T-cell assay, identified single-guide (sg)RNAs targeting EGFR that sensitized tumor cells to T cell–mediated killing. Combination of PD-1 blockade with EGFR inhibition showed significant synergistic efficacy in a syngeneic model, further validating EGFR inhibitors as immunomodulatory agents that enhance checkpoint blockade. This assay can be screened in high-throughput with small-molecule libraries and genome-wide CRISPR/Cas9 libraries to identify both compounds and target genes, respectively, that enhance or inhibit T-cell recognition and killing of tumor cells. Retrospective analyses of squamous-cell head and neck cancer (SCCHN) patients treated with the combination of afatinib and pembrolizumab demonstrated a rate of clinical activity exceeding that of each single agent. Prospective clinical trials evaluating the combination of an EGFR inhibitor and PD-1 blockade should be conducted.

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Introduction

With the FDA approval of immune-checkpoint blocking antibodies, initially targeting CTLA-4 in melanoma (1) and then for PD-1/PD-L1 in melanoma (2), non–small cell lung cancer (NSCLC; ref. 3), head and neck cancer (4), and others (5–7), the field of medical oncology has experienced a paradigm shift in treatment modalities. Combination CTLA-4 and PD-1/PD-L1 blocking antibodies have exhibited synergistic efficacy (2, 8), and numerous trials and preclinical development pipelines are ongoing that utilize antibodies that block one or both of these immune checkpoints in combination with additional checkpoint-blocking antibodies (LAG-3, TIM-3, TIGIT, and B7-H3) or agonistic monoclonal antibodies (4–BB,OX-40,GITR,CD40,andICOS). However, despite all these approaches, not all patients benefit from immunotherapy and, as such, additional therapeutic strategies to enhance the effects of immunotherapy are needed. There is increasing interest in combination therapies that leverage existing technologies to increase the immunogenicity of solid tumors and augment immunotherapeutics, such as anti-PD-1/PD-L1, that are increasingly being viewed as foundational reagents in the medical oncology field. Radiotherapy (9), chemotherapy (10, 11), and targeted agents, such as inhibitors of HDACs (NCT02619253,NCT02437136),BRAF(NCT02818023),andVEGF(NCT00790010),have been or are currently being tested clinically in combination with immune-checkpoint blockade and have been shown to increase response rates. The repurposing of existing conventional therapeutics for combination with checkpoint blockade is an attractive strategy, given the preexisting pharmacodynamic/pharmacokinetic and toxicology properties of such compounds. It was our goal to develop an assay that could be utilized to screen compound libraries in high...
throughput for identification of immunomodulatory features. We engineered a target tumor cell line to express firefly luciferase and a model antigen. We proceeded to coculture these target cells with transgenic CD8+ T cells recognizing the model antigen, such that modulation of antigen-specific, T cell–mediated killing could be assessed by luminescence readout and would identify candidate compounds with immunomodulatory properties. The screen identified the epidermal growth factor receptor (EGFR) as an unappreciated immune-ontology target whose inhibition enhanced anti–PD-1 immunotherapy.

Materials and Methods

Cell lines

ID8 were obtained from the laboratory of Gordon Freeman (DFCI) in 2014, MC38 were purchased from ATCC in 2015, 293T were purchased from Invitrogen in 2011, and the KrasG12D;p53-/-- (KP) cell line was derived in-house from the mouse model (12) in 2016. All cell lines were confirmed to be mycoplasma negative by Charles River Research Animal Diagnostic Services using standard Quantitative Fluorescence PCR (QF-PCR) protocol. Cell lines were authenticated by short-tandem repeat profiling. Only cell lines of <20 passages were used for experiments.

Generation of the luciferized ID8 cell lines

A firefly luciferase-OVA fusion cassette was cloned from the Lenti-LucOS vector as previously described (13) using two-step PCR (14) with primers as follows: attL1 forward: 5′-AGGCTTCCCTGAGGACCATGGAAGCGCGCGCAGCC-3′; attL2 reverse: 5′-AAAAGCATGGTCTCGAGCTAGCTAGGCGCGCTTACAG-3′; attL1-T1 forward: 5′-CCCCCATGAGAACATGTTTATAACTGAAAAACCTCTCCTGAGCGACCTG-3′; attL2-T1 reverse: 5′-GGGGGATTAGCAATGCTTTC-3′. PCR products containing the lucOS open reading frame (ORF) were then used to recombine into the plVX-IRES-Neo lentiviral vector (Clontech) using Gateway LR Clonase II (Thermo Fisher). A renilla luciferase vector was linearized and cloned using NotI and then inserted into the plVX-IRES-Neo lentiviral vector. Plasmids were transfected into One Shot OmniMAX 2 competent cells according to the manufacturer’s protocol (Thermo Fisher). Clones were miniprepped (Qiagen), genotyped by PCR, sequence-verified, and transiently transfected into 293T cells to assess firefly luciferase expression. Positive clones were cotransfected into 293T cells along with d8.9 and VSV-G packaging plasmids (Addgene). ID8-Cas9 cells were transduced with plLVx-lucOS-IRES-Neo or plLVx-rLuc-IRES-Neo vectors and placed under G418 selection for 7 days. Viral production and ID8 spin-fection were conducted according to the Broad Institute’s lentiviral production guidelines (15). Clonal cell lines of “lucOS” and “rLuc” were generated by limiting dilution, expanded for 3 to 4 weeks under puromycin and G418 selection to obtain sufficient cell numbers, confirmed for luciferase and OVA expression. KrasG12D;p53-/-- (KP) cell lines were transduced to express lucOS or rLuc constructs utilizing the same protocols.

Harvesting and activation of OT-I T cells

C57BL/6-Tg(TcrTcrb)1100Mjb/J OT-1 mice (stock 003831; Jackson labs) were bred in-house. Eight- to 12-week-old mice were sacrificed, and spleens were harvested by mechanical separation through a 40-μm filter. Red blood cells were lysed using 1× RBC lysis buffer (BioLegend). Splenic single-cell suspensions were resuspended in TruStain fcX (anti-mouse CD16/32; BioLegend) FCR block diluted 1:100 in FACS buffer (PBS plus 2% FBS) and incubated on ice for 15 minutes. CD8+ OT-I T cells were stained with mouse CD8 (Ly-2) microBeads for 20 minutes, washed with FACS buffer, and isolated using magnetic separation and LS columns according to manufacturer’s protocol (kit# 130-049-401; Miltenyi Biotech). CD8+ T cells were eluted into RPMI (Life Technologies) containing 10% FBS (HyClone), penicillin (100 units/mL), and streptomycin (100 μg/mL; Life Technologies). OT-I CD8+ T cells were then activated with Dynabeads Mouse T-Activator CD3/CD28 beads (Life Technologies) for 24 hours before addition to lucOS/rLuc cocultures. OT-I CD8+ T-cell purity of >95% was confirmed by flow cytometry.

OT-I CTL assay

Ten thousand ID8-lucOS and 10,000 ID8-rLuc cells were plated in 100 μL of cell culture media (DMEM plus 10% FBS, penicillin (100 units/mL), and streptomycin (100 μg/mL) in solid white, flat-bottomed, tissue culture-treated 96-well plates (Thermo Fisher). Overnight-stimulated OT-I CD8+ T cells were then added at the designated effector:target (E:T) ratios with or without compounds (Supplementary Table S1) in a total volume of 200 μL and concentration of 1 μmol/L. Plates were incubated for 48 hours at 37°C and 5% CO2. After 48 hours, 100 μL of media were removed prior to the dual-luciferase assay (cATP E2940, Promega). Calculation of the Fluc/Rluc ratio or of percent surviving OVA-expressing target cells ([Fluc + OT-I/Rluc + OT-I]/[avg Fluc no OT-I/avg Rluc no OT-I] × 100) was utilized where indicated for analysis of compound performance.

Briefly, 50 μL of Dual-Glo luciferase buffer (16) was added to wells and incubated for 30 minutes at room temperature before analysis of firefly luminescence. Dual-Glo Stop and Glo buffer (50 μL) was then added, plates were incubated for 30 minutes, and then analyzed for renilla luminescence on an EnSpire plate reader (PerkinElmer). Two hundred three compounds (Supplementary Table S1; details for LINCS kinase library can be found at http://lincs.hms.harvard.edu/db/sm/) were screened in high throughput at 1 μmol/L final concentration in duplicate in 96-well plates containing both CD8+ OT-1 cytotoxic T lymphocytes (CTL) and ID8 target cells or ID8 target cells only at E:T of 1:1. No compounds were placed in edge wells, and all plates contained multiple DMSO control cells. Performance of compounds was calculated based on ΔΔCt method using ΔΔCt = (FlucDMSO/RlucDMSO)/(Fluccompound/Rluccompound), where values >1 augment CTL killing, values <1 have a negligible immunomodulatory effect, and values <1 inhibit CTL killing. Compounds then reached a certain threshold (top 10%) from high-throughput screen were validated with dose–response curves using the indicated drug concentrations.

OT-I IFNy ELISA

One hundred microliters of the supernatants from the OT-I CTL assays described above were harvested at the 48-hour time point prior to the dual-luciferase assay and analyzed for IFNy secretion with a LEGEND MAX Mouse IFNy ELISA Kit (BioLegend) according to the manufacturer’s protocol. Indicated compounds were tested at 100, 50, 10, 5, and 1 nmol/L with DMSO-only control wells with either no OT-I CTLs or E:T ratios of 1:1 and 2:1. Conditions were assayed with four replicate wells per experiment.
Flow cytometry for MHC class-I expression

100,000 ID8-lucOS, ID8-lucOS sgEFGR KO, KrasG12D.p53–/–, and MC38 cell lines were cultured in 12-well plates in 2 mL of culture media (DMEM) containing 10% FBS, penicillin (100 units/mL), and streptomycin (100 µg/mL) alone or supplemented with recombinant mouse IFN–g (4 ng/mL; BioLegend) for 48 hours. Where indicated, cells were treated with erlotinib, gefitinib, or afatinib at a concentration of 100 nmol/L for the full 48-hour time course. EFGR KO cells had loss of EFGR confirmed by Western blot prior to the assay. Cells were trypsinized, washed, and resuspended in FACS buffer (PBS plus 2% FBS) with H-2Kb/–APC (clone AF6–88.5; BioLegend) at a dilution of 1:100 for 15 minutes on ice, then washed twice prior to analysis on a BD LSRFortessa with FACS DIVA software (BD Biosciences). Data were analyzed using FlowJo software version 10.0.9.

sgEFGR constructs

The top four scoring sgRNA targeting EGFR (sequences listed in Supplementary Table S2) were ordered as oligos from IDT and ligated into BsmBI site in pXPR-sgRNA-GFP-Blast expression vector (Addgene) using Quick Ligation Kit according to the manufacturer’s protocol (cat# M2200S New England Biolabs). Plasmids were transformed into One Shot Stbl3 Chemically Competent E. coli according to the manufacturer’s protocol (cat# C737303 Thermo Fisher). Clones were miniprepped (Qiagen), genotyped by PCR, and sequence-verified. Positive clones were cotransfected into 293T cells along with d8.9 and SG constructs with no OT-I T cells added were also passaged in parallel. Cell cultures were maintained for 72 hours, at which point, live and dead ID8-lucOS cells were harvested for isolation of genomic DNA.

Genomic (g)DNA from cell pellets was extracted using the DNeasy Blood and Tissue Kit (Qiagen) and was concentrated using Genomic DNA Clean and Concentrator (Zymo Research), both according to the manufacturers’ protocol. Twelve micrograms gDNA (250× representation for 8,000 sgRNAs at 6 pg DNA/cell) was amplified using Titanium Taq DNA Polymerase (Clontech) in a one-step PCR reaction with the following parameters: 95°C 1 minute, [95°C 30 seconds, 64°C 30 seconds, 72°C 30 seconds] × 22 cycles, 72°C 5-minute first step with F2/R2 primers. PCR products were verified on a DNA1000 Bioanalyzer (Agilent) and ~350 bp bands were gel-purified using the QIAquick Gel Extraction Kit (Qiagen). PCR products were diluted to 10 ng/µL, pooled, and sequenced on a NextSeq machine (illumina).

In vivo validation

C57Bl/6j mice (stock #000664; Jackson Labs) were challenged subcutaneously with 500,000 MC38 colon cancer cells on their flanks and enrolled on-study when tumors reached 50 mm3. Mice were treated with vehicle plus IgG2a isotype control (10 mg/kg, Bio X Cell), anti–PD-1 (10 mg/kg; clone RMP1-14; Bio X Cell), afatinib (10 mg/kg; Selleck), combination anti–PD-1 (10 mg/kg) and afatinib (10 mg/kg), or combination anti–PD-1 (10 mg/kg), afatinib (10 mg/kg), and anti-CD8α (200 pg, clone 53-6.7; Bio X Cell). Animals received intraperitoneal (i.p.) injections of anti–PD-1 on days 5, 8, and 12 and afatinib on days 6, 7, 8, 9, and 10 (as indicated). Depleting anti–CD8α was administered 2 days prior to first anti–PD-1 treatment. Mice used in experiments were 7 to 8 weeks of age at time of tumor challenge. Endpoint was considered to be when tumors reached a size of 2,000 mm3 or as mandated by institutional guidelines due to development of necrotic lesions. Mice were monitored every 2 to 3 days and tumors measured with digital calipers. All animal studies were conducted in accordance with, as well as with the approval of, the Institutional Animal Care and Use Committee of Dana-Farber/Harvard Cancer Center.

Afatinib and pembrolizumab combination therapy

Retrospective medical record and image review of patients with recurrent and/or metastatic squamous-cell carcinoma of the oral cavity, oropharynx, hypopharynx, or larynx (SCCHN) who received combination afatinib and pembrolizumab at National Taiwan University Hospital between November 1, 2016, and September 30, 2017, with follow-up through March 30, 2018, were evaluated. Exclusion criteria included prior treatment with afatinib, pembrolizumab, or nivolumab as a monotherapy or prior treatment with other anticancer agents in combination with afatinib or pembrolizumab. Disease status was assessed by MRI or CT scan, and responses were reviewed by a radiologist according to RECIST 1.1 criteria. In all, 41 patients were eligible for analysis, with clinical annotation and treatment regimen available in Supplementary Table S3. Patient studies were conducted in accordance with the International Ethical Guidelines for Biomedical Research Involving Human Subjects (CIOMS). Studies were performed after approval by the Institutional Review Board of the National Taiwan University Hospital (NTUH; Taipei, Taiwan). Investigators obtained informed written consent from all subjects.

Data analysis

The following denote statistical significance: *, P < 0.05; **, P < 0.01; ††, P < 0.001. Flank tumor growth curves were analyzed using two-way ANOVA, all bar graphs were analyzed using an unpaired Student t test, and survival experiments used the log-rank Mantel–Cox test for survival analysis. Statistics were calculated using PRISM 7.01 (GraphPad).

Results

Development of OT-I immune-oncology (IO) assay

We selected the ID8 murine serous ovarian carcinoma cell line due to its constitutive expression of MHC class-I, MHC haplotype compatibility with C57Bl/6j mice, and IFN–γ-induced upregulation of PD-L1 (Supplementary Fig. S1). ID8 cells were transduced
with pLVX vectors to express either firefly luciferase and OVA model antigen ("lucOS") or renilla luciferase and no model antigen ("rluc"; Fig. 1A). Target ID8 cell lines were mixed at a 1:1 ratio and cocultured with CD8\(^{+}\) T cells isolated from the spleens of OT-I TCR-transgenic mice. OT-I mice express transgenic TCR\(\alpha\)-V2 and TCR\(\beta\)-V5 genes such that all CD8\(^{+}\) T-cell receptors recognize chicken ovalbumin residues 257 to 264 (SIINFEKL) in the context of H-2K\(\beta\) (17). Target cell–T cell cultures were incubated with compounds for 48 hours and then analyzed by dual-luciferase assays, where changes in firefly signal relative to controls would indicate modulation of T-cell killing by compound treatment (Fig. 1B and C).

The renilla luciferase signal remained relatively constant across wells, regardless of the number of OT-I T cells added to coculture. However, a dramatic loss of firefly luciferase signal with increasing effector:target ratios, indicating that OT-I CD8\(^{+}\) T cells selectively

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**Figure 1.**

OT-I CTL screen design. **A**, ID8-Cas9 serous ovarian carcinoma cell line was transduced with a pLVX vector expressing either firefly luciferase fused to a model antigen peptide or renilla luciferase and no antigen. Clonal cell lines were generated using G418 selection for Neo cassette expression and limiting dilution. **B**, 10,000 ID8-lucOS and 10,000 ID8-rluc were coplated into wells of 96-well tissue culture plates. OT-I TCR-transgenic CD8\(^{+}\) T cells were then plated on top of ID8 cells. Total volume/well was 200 \(\mu\)L and was incubated for 48 hours at 37°C and 5% CO\(2\) prior to analysis by dual-luciferase assay. **C**, OT-I assay was screened in high throughput to evaluate compounds for immunomodulatory effects on antigen-specific tumor cell killing by CTLs. Inclusion of ID8-lucOS and ID8-rluc provided in-plate normalization controls to determine nonspecific growth inhibition or induction of apoptosis by screen compounds.
kill lucOS ID8 cells in an antigen-dependent manner, while sparing rluc ID8 cells (Fig. 2A). Simple calculation of the Fluc/Rluc ratio or of percent surviving OVA-expressing target revealed that an effector:target ratio of ~0.5 was sufficient to observe ~50% killing (Fig. 2B and C). The OT-I IO assay was validated with cyclosporin-A, a well-established inhibitor of CD8+ T-cell effector function (18). As expected, cyclosporin-A inhibited T cell–mediated killing of antigen-expressing target cells in a dose-dependent manner, consistent with published IC50 values (Fig. 2D).

**OT-I IO assay pilot screen and hit validation**

We first screened our OT-I IO assay with a focused library of kinase inhibitors from the Harvard Medical School NIH LINCS Center (Supplementary Table S1). Compounds were screened at a 1 μmol/L concentration, a dose at which nearly a third of the compounds caused nonspecific loss of viability in both antigen-expressing lucOS and control rluc ID8 cells. These compounds were removed from further analysis (Supplementary Fig. S2). Screen results were analyzed based on the normalized firefly/renilla luciferase ratio in DMSO control wells relative to compound-treated wells (Fig. 3). Compounds were considered hits if they fell in the top or bottom 10% of compounds and scored in all replicate plates. Compounds inhibiting OT-I T-cell killing had ratios <1, inert compounds had ratios ~1, and compounds that augmented T-cell killing displayed ratios >1. The CDK9 inhibitor SNS-032, PLK1 inhibitor rigosertib, aurora kinase A inhibitor MLN8054, JAK2 inhibitor AZD-1480, and aurora kinase inhibitor XMD-12-1 (19, 20) all inhibited T cell–mediated target cell lysis (Fig. 3). The GSK-3β inhibitor 6-bromoindirubin and EGFR inhibitor erlotinib were the only two compounds that significantly augmented T-cell killing.

Hits from the initial larger compound screen were validated in the OT-I IO assay with dose–response curves. The JAK2 inhibitor AZD-1480 significantly inhibited T-cell killing with similar...
kinetics to cyclosporin-A, down to low nanomolar concentrations (Fig. 4A and B). However, because of synergistic application with immune-checkpoint blockade, compounds that augmented T-cell killing were of interest. Upon further testing, the GSK-3β inhibitor 6-bromoindirubin and other GSK-3β-specific and GSK-3 inhibitors only modestly augmented T-cell killing (Supplementary Fig. S3). The EGFR inhibitor erlotinib, however, was confirmed to augment T cell–mediated tumor cell lysis (Fig. 4C).

To determine if this effect was erlotinib-specific or EGFR-specific, we also tested gefitinib, an alternative EGFR-specific ATP competitive inhibitor, and afatinib, an irreversible EGFR inhibitor of different chemotype. All three EGFR inhibitors significantly augmented OT-I T-cell killing and, in the case of afatinib, resulted in lysis of almost all OVA-expressing ID8 target cells even at concentrations down to 10 nmol/L (Fig. 4C–E). The mutant-selective EGFR tyrosine kinase inhibitor (TKI) osimertinib exhibited enhanced killing that was inferior to erlotinib, gefitinib, and afatinib (Supplementary Fig. S3). Osimertinib has activity against human keratinocytes (22), leading us to investigate if this mechanism might explain the increased T cell–mediated killing following treatment with EGFR inhibitors in our assay. We observed that erlotinib, gefitinib, and afatinib all significantly increased both basal expression of MHC class-I by ID8 tumor cells and MHC class-I induction by IFNγ secreted by OT-I T cells. In the absence of OT-I effector CD8+ T cells, as expected, sgRNAs targeting essential genes were significantly regulated immune genes), 524 epigenetic regulators, and 34 MHC genes at a coverage of 10 sgRNA per gene and also included 86 immune modulators (immune checkpoints, differentially regulated immune genes), 524 epigenetic regulators, and 34 MHC genes at a coverage of 10 sgRNA per gene and also included 500 nontargeting sgRNAs (Supplementary Table S2). ID8 lucOS cells were transduced with the lentiviral library and cultured at a representation of 500 cells/sgRNA for 72 hours in the presence or absence of OT-I effector CD8+ T cells. In the absence of OT-I T cells, as expected, sgRNAs targeting essential genes were

A CRISPR/Cas9 screen independently identifies EGFR as immunomodulatory

Our OVA-expressing ID8 target cell line was also engineered to constitutively express the Cas9 gene, enabling us to transduce these cells with a sgRNA library and perform the OT-I IO assay in a pooled format. We utilized a library of ~8,000 sgRNAs composed of 87 control genes (essential genes, oncogenes, tumor suppressor genes), 86 immune modulators (immune checkpoints, differentially regulated immune genes), 524 epigenetic regulators, and 34 MHC genes at a coverage of 10 sgRNA per gene and also included 500 nontargeting sgRNAs (Supplementary Table S2). ID8 lucOS cells were transduced with the lentiviral library and cultured at a representation of 500 cells/sgRNA for 72 hours in the presence or absence of OT-I effector CD8+ T cells. In the absence of OT-I T cells, as expected, sgRNAs targeting essential genes were
Figure 4.
Validation of the compound screen results. A, Cyclosporin-A control dose response exhibited predicted inhibition of OT-I T-cell killing. B, AZD 1480 (JAK2 inhibitor) performed similarly to cyclosporin-A. C–E, Erlotinib (EGFR inhibitor) and two other EGFR inhibitors (gefitinib and afatinib) were assayed across a dose range and confirmed screen result that inhibition of EGFR augments antigen-specific T-cell killing. Data presented as raw firefly luciferase (OVA-expressing ID8) values for two different E:T ratios (left), relative firefly luciferase values that account for drug affects ID8 survival irrespective of T cell–mediated killing (middle), and the percentage of survival of ID8-lucOS target cells (right). Experiments were conducted at least twice with similar results and in replicates of four wells per condition. Data for bar graphs calculated using an unpaired Student t test with *, P < 0.05; **, P < 0.01; and ***, P < 0.001 and presented as mean with SD.
preferentially depleted in surviving cells (Fig. 6A–C, red bars). With the addition of OT-I T cells (Fig. 6D), we expected that positive control sgRNAs that targeted immunosuppressive mechanisms, such as PD-L1, would enhance CTL killing, whereas negative control sgRNAs targeting MHC class-I processing and presentation genes should inhibit CTL killing. SgRNAs targeting H2-K1, Tap1, Tap2, and B2m scored as four of our top seven genes enriched in live cells following coculture with OT-I CTLs (Fig. 6E, green bars). SgRNAs targeting our positive control targeting PD-L1 were preferentially depleted in live cells, indicating that loss of this immunosuppressive surface receptor sensitized the ID8 cells to T cell–mediated killing (Fig. 6F, green bar). SgRNAs targeting EGFR were also preferentially depleted from surviving ID8 cells, indicating that loss of EGFR sensitized tumor cells to T cell–mediated killing. EGFR scored as #10 of 731 genes depleted in live cells (Fig. 6F–I). Top-ranking sgRNAs targeting EGFR were used to make individual stable EGFR KO cell lines, which were also sensitized to OT-I T cell–mediated killing across a wide range of effector:target ratios, validating our pooled CRISPR screen results (Supplementary Fig. S5).

EGFR inhibitors synergize with anti–PD-1 therapy

The high antigenicity of the ID8-lucOS model and diffuse nature of the etiology precluded us from using these cells for in vivo validation. We instead utilized the syngeneic MC38 colon due to its well-established, moderate sensitivity to immune-checkpoint blockade (23–25). Mice were implanted with MC38 tumors and then treated with vehicle plus isotype control, afatinib, anti–PD-1, or combination afatinib plus anti–PD-1.

Figure 5.

EGFR inhibition enhances T-cell killing via a tumor cell–intrinsic mechanism. A, ELISA of IFNγ secretion by OT-I CDB8+ T cells cocultured for 48 hours with 10,000 cells at the indicated E:T ratios with the indicated compound treatment. B–E, Expression of MHC class-I by the indicated target tumor cells after inhibition of EGFR with three different compounds of varying chemotypes or sgRNA targeting EGFR. Experiments were conducted at least twice with similar results and in replicates of four wells per condition. Data for bar graphs calculated using an unpaired Student t test with *, P < 0.05; **, P < 0.01; and ***, P < 0.001 and presented as mean with SD.
Combination EGFR inhibition and PD-1 blockade significantly delayed tumor progression relative to vehicle plus isotype control, afatinib, and anti–PD-1 alone (Fig. 7A–C). Combination therapy also conferred significantly improved survival relative to controls ($P = 0.003$), as did anti–PD-1 alone ($P = 0.026$), whereas afatinib single agent had none ($P = 0.487$; Fig. 7D). Combination afatinib and anti–PD-1 was consistent in its tumor inhibition across all 15 mice, and dosing was well-tolerated (Supplementary Fig. S6).

Figure 6. CRISPR/Cas9 screen identifies sgRNAs targeting EGFR as sensitizing tumor cells to T-cell killing. A–C, ID8-lucOS cells alone or (D–H) cocultured at E:T of 1:1 with OT-1 T cells were incubated for 72 hours. After which, genomic DNA was isolated, and sgRNA sequences were deconvoluted by NGS. E and G, sgRNAs targeting genes enriched and (F and H) sgRNAs targeting genes depleted in OT-1 cultures. CRISPR score was defined as the average log2 fold change in abundance of sgRNAs for each gene (10 sgRNA/gene) relative to sgRNA library plasmid pool.
Figure 7. EGFR inhibition enhances efficacy of PD-1 blockade. C57BL/6J mice (15–16 mice/group) were challenged subcutaneously with 500,000 MC38 colon cancer cells on their flanks and enrolled on-study when tumors reached 50 mm³. Mice were treated with anti–PD-1 on days 5, 8, and 12 and afatinib on days 6, 7, 8, 9, and 10 (where indicated). A, Tumor volume of enrolled mice treated with the indicated combinations. B, Tumor volume at day 12 of the study. C, Tumor growth kinetics. D, Survival of mice with the indicated treatments. For survival curves: vehicle vs. afatinib n.s.; vehicle vs. anti–PD-1 P = 0.013; vehicle vs. combo P = 0.003; afatinib vs. anti–PD-1 P = 0.069; afatinib vs. combo P = 0.017; anti–PD-1 vs. combo n.s.; vehicle vs. combo CD8-depleted n.s.; afatinib vs. combo CD8-depleted n.s.; anti–PD-1 vs. combo CD8-depleted P = 0.028. E and F, Response to afatinib and pembrolizumab combination therapy in retrospective cohort of patients with SCCHN. Data presented as (E) pre- and posttreatment scans of selected responders and (F) waterfall plot of % tumor volume change from baseline in response to treatment. Flank tumor growth curves were analyzed using two-way ANOVA, bar graphs were analyzed using unpaired Student t test, and survival experiments used the log-rank Mantel-Cox test for survival analysis. All indicated with * P < 0.05; ** P < 0.01; *** P < 0.001.
Therapeutic efficacy of the combination treatment was lost when CD8+ T cells were depleted (Fig. 7A–D, orange group), confirming that the effect was immune-mediated. We, therefore, concluded that combination PD-1 blockade and EGFR pharmacologic inhibition constitutes a synergistic immunotherapy.

We compiled a retrospective cohort of 41 relapsed/metastatic squamous-cell carcinoma of the head and neck (SCCHN) patients who received combination afatinib and the PD-1 antibody pembrolizumab (Fig. 7E and F) at the National Taiwan University Hospital between November 2016 and September 2017. Combination therapy resulted in an overall response rate (ORR) of 58.5% by RECIST criteria and an average tumor size reduction of 82.9%, without associated increased toxicity (Fig. 7E and F; Supplementary Table S3). This is compared with reported ORR of 16% to pembrolizumab monotherapy (8) and ORR of 10% to afatinib monotherapy (26) in SCCHN patients. These clinical observations support our preclinical findings demonstrating a synergistic effect of EGFR inhibitors with anti–PD-1 inhibitors.

**Discussion**

We created a high-throughput screening assay that can be used to identify both drug candidates (plate-based compound screen) and targets (pooled CRISPR/Cas9 screen). Prior studies have paired target cells expressing a model antigen with CD8+ T cells expressing antigen-specific T-cell receptors with the intent to identify tumor cell–intrinsic immunomodulatory genes (27–29). These studies elucidate mechanisms conferring resistance to immune pressure, and our results were concordant, whether from the compound screen (JAK2 inhibitor AZD1480) or CRISPR/Cas9 screen (H2-K1, Tap1, Tap2, and B2m). However, the studies focused on the fundamental biology and specific pathways that tumor cells often mutate or downregulate to evade T-cell recognition and killing, and our work focused on the opposite end: genes that sensitize tumor cells to CD8+ T-cell–mediated killing.

EGFR was a logical, if initially unexpected hit. EGFR has previously been shown to antagonize HLA class-I expression via suppression of STAT1 in head and neck cancer patients treated with cetuximab (30). Cetuximab-mediated inhibition of EGFR signaling was associated with enhanced IFNγ receptor 1 (IFNAR) expression, which, through STAT1-dependent signaling, enhanced IFNγ-induced expression of HLA class-I and TAP1/2. In another study, pharmacologic inhibitors of EGFR and cetuximab were shown to upregulate basal and IFNγ-induced expression of class I and II in human keratinocytes. The same study provided in vivo validation where patients already receiving erlotinib or cetuximab consented to skin biopsies, and on-treatment elevation in HLA mRNA was demonstrated (22). A genome-wide CRISPR screen characterizing mechanisms of tumor cell immune evasion has identified SOX10 as a top hit, which conferred resistance to T cell–mediated killing commensurate with B2m, HLA-A, and Tap1 (29). This would plausibly implicate an EGFR-related mechanism, as knockdown of SOX10 in human melanoma was previously shown (31) to result in high expression of EGFR, which damped antigen processing and presentation, leading to immune escape.

Any modulation of antigen presentation or tumor cell stress is likely to affect NK cell involvement in the antitumor immune response. Pharmacologic inhibition of EGFR with gefitinib or silencing with siRNA increases expression of MHC-I in the PC9 mutEGFR T790M human NSCLC cell line, which is consistent with our data, and downregulates expression of NKG2D ligands MICB and ULBP-2/5/6 (32). Subsequently, gefitinib attenuates NK cell–mediated lysis of tumor cells. In another study, however, EGFR inhibition with gefitinib enhanced NK cell–mediated cytotoxicity of L858R + T790M mutEGFR tumor cells via upregulation of NKG2D ligands MICA, ULBP1, and ULBP2 (33). EGFR inhibition could potentially enhance or inhibit NK cell recognition of tumor cells by modulation of stress ligands recognized by activating NK cell receptors and through KIR-mediated missing-self recognition that is dependent upon expression of MHC class-I (34). It is possible that there are alternative mechanisms of EGFR inhibitor–mediated immunomodulatory function that involves NK cells.

The immunologic contribution of oncogenic EGFR has been explored clinically (35) and preclinically (36) but mostly as it relates to its regulation of PD-L1 expression in tumor cells. This led to the rational hypothesis that addition of PD-1/PD-L1 blocking antibodies might improve EGFR TKI in EGFR-mutant lung cancer by activating the immune infiltrate otherwise suppressed by secondary mechanisms downstream of aberrant EGFR signaling. Therefore, evaluation of EGFR TKIs with anti–PD-1 therapies has mostly been limited to EGFR-mutant lung cancer. There are currently two clinical trials exploring this combination: nivolumab plus EGFR816 (NCT02323126) and nivolumab plus erlotinib (NCT01454102). A trial of osimertinib, a mutant-selective EGFR inhibitor, combined with the PD-1 inhibitor durvalumab in patients with T790M EGFR-mutant lung cancer, was stopped due to toxicity (NCT02454933). EGFR TKIs are the standard of care for lung cancers harboring EGFR activating mutations but exhibit minimal therapeutic efficacy in wild-type EGFR NSCLC (37, 38), colorectal cancer (39), and SCCHN (26). Only afatinib has an approval in a wild-type EGFR setting. Yet, EGFR-mutant lung cancer, the largest cohort of patients treated with EGFR inhibitors, may not be an ideal setting in which positive immunomodulatory properties would necessarily be noticed, largely due to the immunologically “cold” nature of the disease, as our group and others have shown (40). The data presented above confirming EGFR as an IO target was conducted in three distinct wild-type EGFR models. This suggests that, whether through its regulation of PD-1 or basal and IFNγ-induced antigen processing and presentation, inhibition of EGFR may be broadly efficacious across mutant EGFR and wild-type EGFR cancers. The oncogenic properties of EGFR are well established, but our work supports the classification of EGFR as an IO target. Given the FDA approval of multiple pharmacologic and biologic inhibitors of EGFR and their established clinical application, inclusion of inhibitors to non-mutated EGFR is an attractive approach to amplify the immunogenicity of tumors treated with immune-checkpoint blockade. Our human data in SCCHN are evidence for the utility of this approach. The retrospective clinical data, combined with the synergistic effect of afatinib and anti–PD-1 in our in vivo model, suggested that the combination of immune-checkpoint blockade with EGFR TKI may have a therapeutic benefit in wild-type EGFR cancers. Although our clinical analysis was conducted retrospectively, the findings suggest that EGFR TKIs and anti–PD-1 agents should be evaluated in prospective clinical trials, specifically in wild-type EGFR cancers.

EGFR TKI trials report high incidences of adverse events, such as skin rashes, in up to 66% to 90% of patients (37–39, 41). There is ample evidence both in this paper and in published literature to support the assertion that these drugs induce upregulation of...
this could potentially cause aberrant T-cell recognition of self-antigen. Breaking of tolerance may explain the high rates of toxicity observed with EGFR TKI, and they may be immune-mediated. Immune activation may also explain the therapeutic benefit observed in wild-type EGFR lung and colorectal patients treated with EGFR TKI and, consistent with this hypothesis, the benefit of EGFR TKIs is often greater in patients who develop a skin rash (37, 38). Adverse events are likely to remain consistent, if not become exacerbated, by combination with immune-checkpoint blockade. However, this was a retrospective analysis and not a prospective clinical trial. If toxicity is limiting in future combination trials, this could be potentially mitigated by using EGFR inhibitors at lower dosages or by evaluating different treatment schedules, including sequencing or intermittent dosing of EGFR TKIs.

We present here an assay for high-throughput screening that can be utilized to identify novel immunomodulatory therapeutics and current drugs that would logically be expected to augment immune-checkpoint blockade and other developing immunotherapies. As currently designed, one OT-I mouse spleen with a routine harvest of 10 to 12 million CD8+ T cells is sufficient to plate 10 to 12 96-well assay plates, rendering analysis of compound libraries in the hundreds to thousands feasible. Our initial screen identified EGFR as a target that sensitizes tumor cells to CD8+ T cell–mediated killing, a result that was confirmed in two different murine tumor cell lines and independently validated in a pooled CRISPR/Cas9 screen. Inhibition of EGFR with afatinib enhanced anti–PD-1 therapeutic efficacy in vivo in the MC38 syngeneic colon cancer model and in human SCCHN patients. The CTL OT-I assay is a tool to rationally identify promising drug combinations to enhance immunotherapy, which is rapidly becoming a cornerstone of medical oncology.

References


Disclosure of Potential Conflicts of Interest

P.A. Jänne reports receiving a commercial research grant from AstraZeneca and Boehringer Ingelheim and is a consultant/advisory board member for AstraZeneca, Boehringer Ingelheim, Pfizer, and Roche/Gerentech. No potential conflicts of interest were disclosed by the other authors.

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A High-Throughput Immune-Oncology Screen Identifies EGFR Inhibitors as Potent Enhancers of Antigen-Specific Cytotoxic T-lymphocyte Tumor Cell Killing

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