Targeting Triple-Negative Breast Cancer with Combination Therapy of EGFR CAR T Cells and CDK7 Inhibition

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ABSTRACT

EGFR-targeted chimeric antigen receptor (CAR) T cells are potent and specific in suppressing the growth of triple-negative breast cancer (TNBC) in vitro and in vivo. However, in this study, a subset of mice soon acquired resistance, which limits the potential use of EGFR CAR T cells. We aimed to find a way to overcome the observed resistance. Transcriptomic analysis results revealed that EGFR CAR T-cell treatment induced a set of immunosuppressive genes, presumably through IFN signaling, in EGFR CAR T-cell–resistant TNBC tumors. The EGFR CAR T-cell–induced immunosuppressive genes were associated with EGFR CAR T-cell–activated enhancers and were especially sensitive to THZ1, a CDK7 inhibitor we screened out of a panel of small molecules targeting epigenetic modulators. Accordingly, combination therapy with THZ1 and EGFR CAR T cells suppressed immune resistance, tumor growth, and metastasis in TNBC tumor models, including human MDA-MB-231 cell–derived and TNBC patient–derived xenografts, and mouse EMT6 cell–derived allografts. Taken together, we demonstrated that transcriptional modulation using epigenetic inhibitors could overcome CAR T-cell therapy–induced immune resistance, thus providing a therapeutic avenue for treating TNBC in the clinic.

Introduction

Triple-negative breast cancer (TNBC) comprises 10% to 20% of all breast cancers and is associated with an aggressive phenotype and a high incidence of recurrence (1). To date, there are no approved targeted therapies for TNBC. Proof-of-principle studies have indicated the potential benefits of immunotherapy (2). Antibodies targeting programmed cell death protein 1 (PD-1) and programmed death ligand 1 (PD-L1) have been approved for clinical use (3). Adoptive cell therapy, particularly chimeric antigen receptor (CAR)–modified T-cell therapy, has gained much attention in the past decade (4). Previous studies have demonstrated that EGFR is a potential therapeutic target for TNBC (5). We and others have reported that third-generation EGFR CAR T cells exhibit potent and specific cytotoxicity against TNBC (6, 7).

Despite the unprecedented, durable response rates observed, the majority of patients do not benefit from immunotherapies (primary resistance), and some responders relapse after treatment (acquired resistance; ref. 8). The immune system can paradoxically constrain or promote tumor development and progression. This process is referred to as cancer immunoediting and, in its most complex form, proceeds through three phases: elimination, equilibrium, and escape (9). Both intrinsic and extrinsic factors in tumor cells weigh into this balance (8). Therefore, curative immunotherapy must not only break immunotolerance and generate responses to tumor antigens, but also circumvent the evolving barrage of acquired escape mechanisms (10).

The commonly cited impediments to effective CAR T-cell therapy include the loss or modulation of the target antigen, lack of CAR T-cell persistence, cytokine-release toxicity, and product manufacturing failures (11). However, tumor-associated impediments that lead to resistance to CAR T-cell therapy remain elusive. Effective and prolonged immunotherapy requires synergy to eliminate impediments from both intrinsic and extrinsic factors of tumor cells (12). Emerging evidence indicates that epigenetic modulation can robustly sensitize patients to immunotherapy. Epigenetic modifiers, such as inhibitors of histone deacetylase, DNA methyltransferase, lysine-specific histone demethylase 1, enhancer of zeste homolog 2, and bromodomain and extraternal (BET) proteins, have been shown to regulate the presentation and generation of neoantigens and immune checkpoints, secretion of cytokines, and activation of immune cells (13, 14). Enhancers, particularly superenhancers, which participate in regulating the expression of key genes in tumor cells, are particularly sensitive to treatment and show great promise for therapeutic intervention. Enhancer-associated transcription depends on a plethora of transcription factors and cofactors (15). Accumulating evidence suggests that BET inhibition (BETi) can suppress PD-L1 transcription in cancer and immune cells to boost anticancer immune responses (16, 17). However, the function of other proteins associated with enhancers in modulating immune responses remains to be characterized.

In this study, we report that TNBC tumors in one third of mice acquired resistance to third-generation EGFR CAR T-cell treatment. Transcriptomic analysis of EGFR CAR T-cell–resistant tumors revealed the activation of a large set of genes associated...
with immune suppression, which were presumably activated by IFNγ released by CAR T cells. These CAR T-cell–induced immunosuppressive genes were associated with CAR T-cell–activated enhancers and were especially sensitive to THZ1, a CDK7 inhibitor. Accordingly, combination therapy with THZ1 and EGFR CAR T cells suppressed immune resistance, tumor growth, and metastasis of TNBC in mice.

**Materials and Methods**

**Cell lines and cell culture**

HEK293T and EMT6 cells were purchased from the American Type Culture Collection in 2018, and MDA-MB-231 and MDA-MB-468 cells were purchased from the Cell Bank of the Chinese Academy of Sciences in 2018. HEK293T and MDA-MB-231 cells were maintained in DMEM high glucose (Biological Industries) supplemented with 10% heat-inactivated FBS (Gibco). MDA-MB-468 cells were maintained in RPMI 1640 (Biological Industries) medium supplemented with 10% heat-inactivated FBS, and EMT6 cells were maintained in Waymouth’s Medium (Gibco) supplemented with 15% heat-inactivated FBS. All cells were maintained in a humidified incubator with 5% CO2 at 37°C. Cell lines were not authenticated since purchase and were cultured for fewer than 10 passages. Cell lines were routinely tested for Mycoplasma using a Mycoplasma contamination detection kit (rep-p1, InvivoGen).

**Generation of stable cell lines**

Full-length human PD-L1 (NM_011413.4), HVEM (NM_001297602), and IDO1 (NM_001264.6) cDNAs were first cloned into the lentiviral vector pCDH-CMV-MCS-EF1-Puro (VT1480, Youbio). HEK293T cells were then transfected with the plasmid mixture of the lentiviral and packaging vectors, psPAX2 (12260, Addgene) and pMD2.G (12259, Addgene), using polyethyleneimine (Mw 40,000, 24765-2, Polysciences) following the manufacturer’s instructions. Lentivirus-containing supernatants were collected after 48 hours of transfection. MDA-MB-231 cells were then transduced with these lentiviruses, and positive clones were selected by puromycin selection. Knockdown of STAT1 and CDK7 was verified by Sangon were cloned into the lentiviral vector (lentiCRISPR CAG-3

**Animal experiments**

For combination treatment with EGFR CAR T cells and THZ1 (CDK7 inhibitor), the mouse EGFR-targeted CAR construct (EGFR mCAR) consisted of an anti-human EGFR single-chain variable fragment (scFv) derived from cetuximab, spacer, a transmembrane domain derived from murine CD28, and intracellular signaling domains derived from murine CD28, 4-1BB, and CD3ζ. Sequence alignment between human and mouse EGFR revealed 87% similarity for the scFv region. This EGFR mCAR sequence was cloned into the MSCV-ires-GFP retroviral vector (20672, Addgene). HEK293T cells were transduced with a plasmid mixture of retroviral vectors and pCl-Eco plasmid (12371, Addgene) using polyethylenimine following the manufacturer’s instructions for 60 hours before collecting supernatants, which were further concentrated using Amicon Ultra-15 Centrifugal Filters (100 kDa, UFC91096, Merck). Concentrated retroviruses were stored at −80°C.

Spleens of female BALB/c mice ages 6 to 12 weeks (Shanghai SLAC Laboratory Animal Center) were disrupted using a 70 μm strainer to prepare a single-cell suspension. Murine T cells were then isolated using the EasySep Mouse T Cell Isolation Kit (19851, STEMCELL Technologies) according to the manufacturer’s instructions, yielding over 90% purity of T cells. For activation of T cells, isolated T cells suspended in X-VIVO medium (04-418Q, LONZA) were stimulated on plates coated with mouse CD3 (1 μg/mL, 100302, BioLegend) and mouse CD28 antibodies (2 μg/mL, 102102, BioLegend) for 24 hours. Activated murine T cells were transduced with concentrated retroviruses in 24-well plates at a multiplicity of infection of 50 for 1 hour (37°C, 2,000 × g) and were rested for 48 hours in X-VIVO medium supplemented with mouse IL2 (40 ng/mL, S1061-MNAE, Sino Biological) and IL7 (5 ng/mL, S0117-MNAE, Sino Biological) for 3 days before use.

**Patient samples and generation of a patient-derived xenograft model**

A TNBC tumor samples were collected with the understanding and informed written consent from a patient with TNBC, and the study was conducted in accordance with the Declaration of Helsinki and approved by the Review Board and Ethics Committee of Shanghai Cancer Hospital. Fresh tumor samples were collected in DMEM high-glucose medium on ice, cut into pieces, and digested into single tumor cells with collagenase (300 U/mL) and hyaluronidase (100 U/mL, 07912, STEMCELL Technologies), which were then transfected s.c. into female nude mice ages 6 to 12 weeks (Shanghai SLAC Laboratory Animal Center). Mice were sacrificed when the tumor size reached 1,500 mm³. Tumors were collected and retransplanted into nude mice for in vivo passage. After the establishment of patient-derived xenograft (PDX), tumors were cut into pieces, digested into single cells, and cryopreserved at −80°C in storage buffer (85% FBS and 15% DMSO).


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inhibitor; HT-80013, MedChemExpress), tumors were allowed to grow until the mean flux reached approximately $1 \times 10^6$ p/s/cm$^2$/sr (day 0). Mice were then randomly assigned to four groups (3 mice/group): CTL T cells ($5.0 \times 10^5$ cells/injection, i.v. injection), EGFR CAR T cells ($5.0 \times 10^5$ cells/injection, i.v. injection), and THZ1 (10 mg/kg, i.p. injection) combined with EGFR CAR T cells ($5.0 \times 10^5$ cells/injection, i.v. injection). Mice were injected with CTL T cells or CAR T cells once every other day, following THZ1 treatment for 65 days and observed for 130 days. Mice were sacrificed before the tumor burden exceeded the limit ($\sim 1,500$ mm$^3$). Tissue samples were resected, formalin-fixed/paraffin-embedded, and stored at $-20^\circ$C before histopathologic analysis (see below).

For rechallenge experiments, SCID mice inoculated with MDA-MB-231-fluc cells ($5.0 \times 10^5$ cells/mouse) were first treated with THZ1 and EGFR CAR T cells as described above. Cured mice and age-matched healthy SCID mice were s.c. injected with MDA-MB-231-fluc cells ($5.0 \times 10^5$ cells/mouse, 5 mice/group) after 65 days of treatment. Mice were sacrificed before the tumor burden exceeded the limit ($\sim 1,500$ mm$^3$).

If tumor volume started and continued to increase at a similar rate as the control group with CAR T-cell treatment, and exhibited no response to CAR T-cell treatment when reimplanted into SCID mice, the tumors were considered resistant. Meanwhile, tumors in mice continuously showing decreased volume when treated with CAR T cells were considered sensitive. To treat CAR T-cell–resistant tumors, fresh tumor tissues were isolated from CAR T-cell–resistant tumor-bearing mice after 31 days of treatment. After lysis of erythrocytes by Red Blood Cell Lysis Buffer (R1010, Solarbio), cells were washed with PBS and digested into single-cell suspensions with collagenase (300 U/mL) and hyaluronidase (100 U/mL; #07912, STEMCELL Technologies) at 37°C according to the manufacturer’s instructions. Single tumor cells ($2 \times 10^5$) were mixed with Matrigel (354243, BD Biosciences) and transplanted s.c. into SCID mice ages 6 to 12 weeks. When tumors reached 100 to 200 mm$^3$ in size (day 0), mice were randomly assigned to four groups (5 mice/group) and treated with CTL T or EGFR CAR T cells ($5.0 \times 10^5$ cells/dose, i.v. injection) in the presence or absence of THZ1 (10 mg/kg/dose, i.p. injection) as described above. Treatment lasted 14 days, and the observation continued until day 35. Peripheral blood was collected (retro-orbital) described above. Treatment lasted 29 days, and the observation continued until day 26.

Tumor progression was monitored daily by bioluminescence using the Xenogen IVIS Lumina imaging system (Caliper Life Sciences), and tumor size was measured daily with a caliper. Each mouse was injected i.p. with beetle luciferin (1.5 mg; E1605, Promega) and then imaged 6 to 8 minutes later with an exposure time of 3 minutes. Luminescence images were analyzed using the Living Image software (Caliper Life Sciences). Tumor volume ($V$) was calculated using the formula: $V = \frac{1}{2} (length \times width^2)$. Mice were weighed every 1 to 3 days after infusion. The survival of mice was monitored daily. All animal experiments were conducted in accordance with a protocol approved by the Animal Care and Use Committee of Xiamen University.

Cytotoxicity assay

Cytotoxicity assays were performed using the xCELLigence Real-time Cell Analyzer (RTCA) System (ACEA Biosciences) as described previously (6). MDA-MB-231 or EMT6 cells were seeded and cultured for 24 hours. Control T cells or EGFR CAR T cells with or without THZ1 (250 nmol/L) were added to the RTCA unit at different ratios. Impedance signals were recorded for 72 hours at 5-minute intervals.

Copy-number analysis

To assess the persistence of the CAR T cells in recipient mice, peripheral blood mononuclear cells (PBMC) of SCID mice were collected, and genomic DNA (gDNA) was extracted using a DNeasy Blood & Tissue Kit (69506, QIAGEN). gDNA (20 ng) was then subjected to qRT-PCR analysis using a primer pair targeting the junction of the CD137 domain and adjacent CD3$\gamma$ chain (forward primer: 5'-GAAGAAGGAGGAGTTGGAATC-3'; reverse primer: 5'-TCTCTCTCTGTGTCTCAGATT-3') on an AriaMx Real-Time PCR System Module (G8831A, Agilent Technologies). gDNA extracted from PBMCs in CTL T–treated mice was used as control. Standard curves were prepared using serial dilutions of the CAR plasmid, starting at $10^5$ copies/mL.

Flow cytometry

Surface expression of EGFR on MDA-MB-231 cells and TNBC PDX was detected using the phycocerythrin (PE)-conjugated mouse anti-human EGFR antibody (555997, BD Biosciences). Murine T cells were isolated from the spleens of BALB/c mice as described above. Surface expression of the mCAR construct on murine T cells was detected using a Myc-Tag (9B11) mouse mAb (PE-conjugated; 3739, Cell Signaling Technology). CD16/CD32 mAb (93)(FcR blocker 14-0161-82, ebioscience) was used to block nonspecific staining. Zombie Aqua Fixable Viability Kit (423102, BioLegend) was added to exclude dead cells. After using forward scatter area/side scatter area (FSC-A/SSC-A) to separate cells from debris, forward scatter area/forward scatter height (FSC-A/FSC-H) were used to gate single cells from doublets. Zombie-negative cells were further gated as live cells. Fluorescence was assessed using an Attune NxT Flow Cytometer (Thermo Fisher Scientific), and the data were analyzed using FlowJo vX v0.7 (BD Biosciences).

ELISA

Mouse serum samples (20 µL) were diluted and detected for the presence of IL6 and Indoleamine 2,3-dioxigenase 1 (IDO1) using the Human IL6 ELISA Kit (EH004-96, ExCell Bio) and Human Indoleamine 2,3-dioxigenase/IDO ELISA Kit (Colorimetric; NBP2-62765, Novus), respectively, following the manufacturer’s instructions.
absorbance was assessed using an Enzyme-label analyzer (infinite F50, Tecan). The concentrations were evaluated using a standard curve by plotting the absorbance (y-axis) against the protein concentration (x-axis).

**Histopathologic analysis**

Tissue samples from mice were resected, formalin-fixed, and paraffin-embedded. Tissue sections were deparaffinized and rehydrated before staining. For IHC staining, EDTA antigen retrieval solution (MSV-0098, Maxim Biotechnologies) and UltraSensitiveTM SP (Mouse/Rabbit) IHC Kit (Kit-9730, Maxim Biotechnologies) were used according to the manufacturer's instructions. Primary antibodies against human Ki67 (Kit-0005, Maxim Biotechnologies), human EGFR (RMA-0689, Maxim Biotechnologies), human CD8 (ab17147, Abcam), human PD-L1 (ab213524, Abcam), human PD-L2 (ab187662, Abcam), human IL6 (ab239482, Abcam), and human IL8 (ab18672, Abcam) were incubated at 4°C overnight. Stained tissue sections were developed using the Diaminobenzidine (DAB) kit (DAB-1031, Maxim Biotechnologies) for 1 minute and counterstained with hematoxylin solution (HHS16, Sigma-Aldrich) for 10 minutes. For hematoxylin and eosin staining, sections were stained with hematoxylin solution (HHS16, Sigma-Aldrich) for 5 minutes and eosin Y solution (318906, Sigma-Aldrich) for 1 minute. For TdT-mediated dUTP nick end labeling (TUNEL) assays, sections were treated with proteinase K (20 μg/mL; 0706, AMRESCO) at 37°C for 20 minutes and then washed in 1× phosphate buffer. The Colorimetric TUNEL Apoptosis Kit (C1098, Beyotime Institute of Biotechnology) was used to detect apoptotic cells according to the manufacturer’s instructions. Histopathologic images were obtained by Olympus BX51 microscope and analyzed using the Olympus cellSens Standard software. The histopathologic images were further converted using ImageJ software to quantify positive expression. Liver and lung metastases were determined by Ki67- and EGFR-positive staining.

**siRNA transfection, RNA isolation, and qRT-PCR**

Control siRNA (siCTL, siN000000-1-1-1) and siRNAs targeting STAT1 (siSTAT1, 5'-CTGGATATTACAGGTTGCAA-3') or CDK7 (sicDK7, 5'-CATACAGGCCTTTCTTACA-3') were synthesized by Ribobio. siRNA transfections were performed using Lipofectamine 2000 Transfection Reagent (11668500, Invitrogen) according to the manufacturer’s protocol. Specifically, MDA-MB-231 cells transfected with siCTRL or siSTAT1 were incubated with or without EGFR CAR T cells at a ratio of 2:1 or IFNγ (10 ng/mL) for 48 hours; MDA-MB-231 or MDA-MB-468 cells transfected with sicCTRL or sicCDK7 were incubated with CAR T cells at a ratio of 2:1 or IFNγ (10 ng/mL) for 48 hours. Total RNA was isolated using the Eastep Super Total RNA Extraction Kit (LS1040, Promega) following the manufacturer’s protocol. Total RNA (1 μg) was subjected to first-strand cDNA synthesis using the GoScript Reverse Transcription System (A5001, Promega), followed by qPCR using an AriaMx Real-Time PCR machine (Agilent Technologies). Beta-actin was used as an internal control. Expression data presented were the normalized value to control samples after normalization to the expression of beta-actin. Primer sequence for each gene is displayed in Supplementary Table S1.

**RNA sequencing**

MDA-MB-231 cells were incubated with CTL T or EGFR CAR T cells at a ratio of 2:1 and incubated with or without THZ1 (250 nmol/L) for 24 hours. T cells in suspension were removed, and the adherent tumor cells were collected. Dead tumor cells were also removed using the Dead Cell Removal Kit (Milltenyi Biotec) before RNA sequencing (RNA-seq) analysis. Tumor tissues from the EGFR CAR T cell–resistant and –sensitive groups (n = 3) were also subjected to RNA-seq analysis (dissociated as indicated above to obtain single-cell suspensions). The Eastep Super Total RNA Extraction Kit (LS1040, Promega) was used for RNA isolation. DNaise I (2 U/μL; 5 μL) was included in the column digestion to ensure RNA quality. RNA library preparation was performed using the NEBNext UltraTM Directional RNA Library Prep Kit for Illumina (E742OL). Paired-end sequencing was performed using an Illumina HiSeq 3000 system. Sequencing reads were aligned to the hg19 RefSeq database using Tophat (http://ccb.jhu.edu/software/tophat/index.shtml). Cuff-diff was used to quantify the expression of RefSeq-annotated genes with the option -M (reads aligned to repetitive regions were masked) and -u (multiple aligned reads were corrected using “rescue method”). Coding genes with fragments per kilobase per million mapped reads (FPKM) larger than 0.5 were included in our analysis. Up- or downregulated genes were determined by the fold change of the gene FPKM between groups. The FPKM of a gene was calculated as mapped reads on exons divided by exon length and the total number of mapped reads. Box plots and heat maps were generated using R software, and statistical significance was determined using the Student t test.

**Chromatin immunoprecipitation coupled with high-throughput sequencing**

For chromatin immunoprecipitation (ChIP) assays, MDA-MB-231 cells were incubated with CTL T cells or EGFR CAR T cells at a ratio of 4:1 and then incubated with or without THZ1 (250 nmol/L) for 24 hours. Alternatively, MDA-MB-231 cells were incubated with or without IFNγ (10 ng/mL) in the presence or absence of THZ1 (250 nmol/L) for 24 hours. T cells in suspension were removed, and the adherent MDA-MB-231 cells were washed twice with PBS and then fixed with 1% formaldehyde (689316, Sigma) for 10 minutes at 25°C. Fixation was stopped by adding glycine (1610718, Bio-Rad; 0.125 mol/L), and the cells were incubated for 5 minutes at room temperature, followed by washing with PBS twice. Cells were lysed in lysis buffer (1% SDS, 10 mol/L EDTA, and 50 mol/mL Tris-HCl), and chromatin DNA was sheared to an average size of 300 to 500 bp by sonication using Picoruptor (Diagenode, P-141006). The resultant protein and DNA mixture (~500 μg) was then diluted with dilution buffer (1% Triton X-100, 2 mol/mL EDTA, 150 mol/mL NaCl, and 20 mol/mL Tris–HCl) and subjected to immunoprecipitation with anti–Pol II (2 μg), anti–Pol II ser5pho (2 μg), and acetylated histone H3 lysine 27 (H3K27Ac, 1.5 μg) antibodies overnight at 4°C, followed by incubation with protein G magnetic beads (0.4 mg/mL, 1614023, Bio-Rad) for an additional 4 hours. After washing, the protein–DNA complex was reversed by heating at 65°C overnight. Immunoprecipitated DNA was purified using the QIAquick PCR Purification Kit (28104, QIAGEN) and subjected to high-throughput sequencing. The following antibodies were used: anti–Pol II (A300-653A, Bethyl Laboratories), anti–Pol II ser5pho (ab5131, Abcam), and anti–H3K27Ac (ab4729, Abcam).

**Immunoblotting**

Immunoblotting was performed as described previously (6). Specifically, cells listed below were subjected to immunoblotting: MDA-MB-231 cells incubated with CTL T or EGFR CAR T cells at a ratio of...
Results

**EGFR CAR T-cell–released IFNγ induces immunosuppressive genes in TNBC cells**

Our previous study demonstrated that third-generation EGFR CAR T cells are potent and specific in suppressing TNBC (6). To further understand the molecular pathways activated by EGFR CAR T cells, RNA-seq in MDA-MB-231 cells revealed that EGFR CAR T-cell treatment altered the expression of a large cohort of genes (Fig. 1A). The IFNγ response was the most enriched hallmark gene set and gene ontology term among genes upregulated by EGFR CAR T-cell treatment, which is consistent with the fact that EGFR CAR T cells secrete high levels of IFNγ when co-incubated with TNBC cells (ref. 6; Fig. 1B; Supplementary Fig. S1A). IFNγ-mediated cellular response is well known to be involved in adaptive immune resistance (6). Immunosuppressive genes, such as CD274 (PD-L1), PDCD1LG2 (PD-L2), TNFSF14 (HVEM), IL6, CXCL8 (IL8), CSF1, CXCL2, IDO1, and IL1B, were significantly induced by EGFR CAR T cells (Fig. 1C, left two columns; refs. 18–24). In addition to the IFNγ/cytokine-mediated signaling pathway, response to endoplasmic reticulum stress, apoptotic signaling pathway, and positive regulation of programmed cell death, which are known to play important roles in antitumorogenesis, were also among the most enriched terms (Fig. 1B; Supplementary Fig. S1A; refs. 25, 26).

To confirm that the primary cellular response induced by EGFR CAR T cells was activated by IFNγ, RNA-seq was performed with IFNγ treatment. The effect of IFNγ on the whole transcriptome correlated with that of EGFR CAR T cells (Supplementary Fig. S1B). A total of 1,090 overlapping genes were found to be induced, which were 46.3% and 57.9% of EGFR CAR T-cell– and IFNγ-induced genes, respectively (Fig. 1D–F). The immunosuppressive genes listed above were among the genes commonly induced by EGFR CAR T cells and IFNγ (Fig. 1C). The induction of these genes was validated, which appeared to be dependent on STAT1, a critical regulator of the IFNγ signaling (Fig. 1G and H). Similar observations were made in MDA-MB-468 cells (Supplementary Fig. S1C and S1D). Taken together, our data suggest that the primary cellular response to EGFR CAR T cells in TNBC was the activation of IFNγ signaling, which induced expression of immunosuppressive genes.

**Immunosuppressive genes are induced in EGFR CAR T-cell–resistant TNBC xenografts**

The activation of immunosuppressive genes suggests that TNBC tumor cells in mice receiving EGFR CAR T-cell treatment might become resistant. Approximately 30 days after treatment, resistance was observed in one third of the mice. Tumors from EGFR CAR T-cell–sensitive and –resistant groups were subjected to RNA-seq analysis (Fig. 2A–D). Compared with sensitive tumors, 262 and 266 genes were up- and downregulated in CAR T-cell–resistant tumors, respectively (Fig. 2C). The IFNγ response was among the top most enriched pathways for upregulated genes, including the immunosuppressive genes (Fig. 2E). Induction of immunosuppressive gene expression was confirmed by qRT-PCR analysis (Fig. 2F).

**EGFR CAR T-cell–induced immunosuppressive genes are associated with enhancers**

The observation that immunosuppressive genes are activated in EGFR CAR T-cell–resistant TNBC tumors led to our hypothesis that blocking such activation might relieve immune resistance. To test this hypothesis, we first characterized the mechanism by which EGFR CAR
Figure 1.
EGFR CAR T cells activate a cohort of immunosuppressive genes presumably through IFNγ in TNBC cells. A, MDA-MB-231 cells were incubated with CTL T or EGFR CAR T cells for 48 hours. T cells in suspension were removed, and the adherent MDA-MB-231 cells were subjected to RNA-seq analysis. Genes up- and downregulated are shown by pie chart [fold change (FC) > 1.5]. B, Hallmark gene set enrichment analysis for genes upregulated. C, The expression (FPKM, log2) of representative immunosuppressive genes in response to EGFR CAR T-cell or IFNγ treatment is shown. D, Overlapping genes induced by EGFR CAR T cells and IFNγ are shown (FC > 1.5). E and F, Heat map (E) and box plot (F) representation of the expression (FPKM, log2) for genes commonly induced by EGFR CAR T cells and IFNγ. Box plot shows median (line), top and bottom quartiles (boxes), and lines extending to highest and lowest observations (whiskers). P values are shown; paired Student’s t test, two-tailed. G and H, MDA-MB-231 cells were transfected with control siRNA (siCTL) or siRNA specific targeting STAT1 (siSTAT1), and then incubated with or without CAR T cells (G) or IFNγ (10 ng/mL; H) for 48 hours. MDA-MB-231 cells were subjected to qRT-PCR analysis. Experiments were repeated three times, and representative data are shown. Statistical significance is shown in Supplementary Table S2. *, P < 0.05; **, P < 0.01; ***, P < 0.001; unpaired Student’s t test, two-tailed.
Figure 2.
Immunosuppressive genes are expressed in EGFR CAR T-cell–resistant TNBC xenografts and are associated with enhancers. A, SCID mice were injected s.c. with MDA-MB-231 cells (1.0 × 10⁶ cells/mouse) and treated with or without CAR T cells (5.0 × 10⁶ cells/injection, 10 mice/group) 2 weeks after (day 0). The treatment lasted for 31 days, when resistance was seen. B, Primary tumor tissues in EGFR CAR T-cell–sensitive or –resistant mice were subjected to RNA-seq (n = 3). Blue points: significantly changed genes (q < 0.05); gray points: unchanged genes. C, Genes up- or downregulated in CAR T-cell–resistant tumors [fold change (FC) > 1.5, q < 0.05]. D, Heat map representation of the expression (FPKM, log₂) for genes up- and downregulated. E, Hallmark gene set analysis for genes upregulated. F, Primary tumor tissues from A were subjected to qRT-PCR analysis. Experiments were repeated three times, and representative data are shown. Statistical significance is shown in Supplementary Table S2. G, MDA-MB-231 cells were incubated with EGFR CAR T cells or IFNγ (10 ng/mL) in the presence or absence of THZ1 (250 nmol/L) for 24 hours. MDA-MB-231 cells were subjected to H3K27Ac ChIP-seq. Genomic distribution of H3K27Ac–occupied sites in the presence of EGFR CAR T cells is shown. TTS, transcription termination site; UTR, untranslated region. H and I, Tag density distribution of H3K27Ac centered on those H3K27Ac sites (± 5,000 bp) in the proximity of EGFR CAR T-cell–induced genes. J and K, Box plot representation of tag density as shown in H/I and L/M. Box plot shows median (line), top and bottom quartiles (boxes), and lines extending to highest and lowest observations (whiskers). P values are shown at the top of the box plot; paired Student t test, two-tailed. L, Box plot shows median (line), top and bottom quartiles (boxes), and lines extending to highest and lowest observations (whiskers). P values are shown at the top of the box plot; paired Student t test, two-tailed. N, and O, H3K27Ac ChIP-seq on selected immunosuppressive gene and cognate enhancer regions.
T cells/IFNγ activated downstream target genes, particularly the immunosuppressive genes. Enhancers, including superenhancers, have been suggested to play a vital role in signaling-induced gene transcriptional activation (27). We hypothesized that EGFR CAR T cells/IFNγ might also activate such an enhancer program. To define the active enhancer landscape upon EGFR CAR T-cell treatment, we performed ChIP sequencing (ChIP-seq) for H3K27Ac, a histone marker decorating both active promoters and enhancers (Fig. 2G). The majority of EGFR CAR T-cell–induced genes were associated with H3K27Ac-occupied enhancers nearby in the presence of CAR T cells. The occupancy of H3K27Ac was significantly higher than that in the group incubated with CTL T, suggesting that they were responsive to EGFR CAR T-cell treatment and might be involved in the activation of the EGFR CAR T-cell–induced genes (Fig. 2H and I). EGFR CAR T-cell–induced active enhancers on representative genes are shown in Fig. 2J and K (Supplementary Fig. S2A). EGFR CAR T-cell–activated enhancers were similarly activated by IFNγ, with similar effects on gene activation (Fig. 2L–O; Supplementary Fig. S2B). Taken together, our data revealed that the EGFR CAR T-cell–activated gene cohort was associated with the activation of nearby enhancers.

**THZ1 suppresses EGFR CAR T-cell–induced immunosuppressive gene activation**

We next sought to interfere with EGFR CAR T-cell–activated enhancers and enhancer–associated immunosuppressive genes, aiming to overcome the associated immune resistance. Epigenetic modifiers, such as CDK7, BRD4, P300, TIP60, MOF, KDM5, and CDK9, are involved in the activation of enhancers and enhancer–associated genes (28–33). The effects of THZ1 (CDK7 inhibitor), IQ1 (BRD4 inhibitor), C646 (P300 inhibitor), MGI49 (TIP60 and MOF inhibitor), KDM5-C70 (KDM5 subfamily inhibitor), or iCDK9 (CDK9 inhibitor) on the CAR T-cell–induced expression of immunosuppressive genes were then tested (Fig. 3A). Among all the inhibitors tested, THZ1 displayed the most consistent and dramatic attenuation of EGFR CAR T-cell–induced gene expression (Fig. 3A). The effects of THZ1 on the protein expression of representative genes were also demonstrated (Fig. 3B).

To extend our observations, RNA-seq results revealed that the expression of 67% of EGFR CAR T-cell–induced genes was attenuated by THZ1 (Fig. 3C–E). The immunosuppressive genes were among the genes that were most affected (Fig. 3F). EGFR CAR T-cell–induced enhancer activation was similarly attenuated, as seen from the occupancy of H3K27Ac (Fig. 2H–K). Similarly, THZ1 treatment attenuated the expression of 47% IFNγ-induced genes, including the immunosuppressive genes (Fig. 3G–J), which was further confirmed by qRT-PCR and immunoblotting (Fig. 3K–N). IFNγ-induced enhancer activation was attenuated (Fig. 2L–O). The inhibitory effects of THZ1 were also demonstrated in another TNBC cell line, MDA-MB-468 (Supplementary Fig. S2C–S2F). The “Chiches cluster” of superenhancer-associated, TNBC-specific genes reported previously was similarly affected by THZ1, which was further validated (Supplementary Fig. S2G and S2H; ref. 29). The effect of THZ1 on EGFR expression was also examined; the expression of membrane EGFR was slightly reduced by THZ1 after incubation for 6 days but did not change further after treatment for a longer duration (Supplementary Fig. S2I). Taken together, THZ1, a CDK7 inhibitor, could suppress EGFR CAR T-cell–induced enhancer and enhancer–associated immunosuppressive gene expression.

**THZ1 inhibits RNA Pol II phosphorylation to suppress immunosuppressive genes**

We next tested whether CDK7, the major target of THZ1, is required for the expression of EGFR CAR T-cell–induced immunosuppressive genes. The requirement of CDK7 for the expression of the set of immunosuppressive genes was demonstrated by qRT-PCR and immunoblotting (Fig. 4A and B; Supplementary Fig. S3A). Similarly, CDK7 was required for the IFNγ-induced expression of representative immunosuppressive genes (Fig. 4C and D). The requirement of CDK7 was also observed in MDA-MB-468 cells (Supplementary Fig. S3B–S3E). CDK7, a component of the general transcription factor TFIIH, phosphorylates the carboxyl-terminal domain of RNA Pol II at serine 5 (RNA Pol II Ser5P) to promote transcription (34). We then tested whether THZ1 inhibited CDK7-mediated Pol II phosphorylation to inhibit the expression of immunosuppressive genes. Upon EGFR CAR T-cell treatment, RNA Pol II occupancy simultaneously increased in both the promoter and gene body regions of the genes induced by EGFR CAR T cells, and this was suppressed by THZ1, suggesting that EGFR CAR T cells regulated the transcriptional initiation of these genes (Fig. 4E). Indeed, the traveling ratio or promoter–proximal pausing index, which is defined as the relative ratio of Pol II density in the promoter–proximal region and the gene body and used to measure transcriptional elongation, exhibited no significant changes upon EGFR CAR T-cell treatment, supporting that EGFR CAR T cell regulated the transcriptional initiation of these genes rather than elongation (Supplementary Fig. S3F). The expression of RNA Pol II Ser5P/o was significantly induced by EGFR CAR T cells, further supporting the EGFR CAR T-cell regulation of transcription initiation (Fig. 4F). Consistent with its inhibitory effects on EGFR CAR T-cell–induced transcriptional activation, THZ1 attenuated EGFR CAR T-cell–induced Pol II and Pol II Ser5P/o (Fig. 4E and F). Similarly, THZ1 attenuated EGFR CAR T-cell–induced RNA Pol II and Pol II Ser5P/o occupancy on EGFR CAR T-cell–activated enhancers (Fig. 4G–J). The effects of THZ1 were shown on representative immunosuppressive genes (Fig. 4K and L; Supplementary Fig. S3G). Taken together, our data revealed that THZ1 inhibited CDK7-mediated RNA Pol II phosphorylation to suppress EGFR CAR T-cell–induced enhancer and cognate gene activation.

**THZ1 improves the efficacy of EGFR CAR T cells in vitro**

The ability of THZ1 to suppress EGFR CAR T-cell–induced immunosuppressive genes prompted us to examine whether cotreatment with THZ1 would improve the efficacy of EGFR CAR T cells in killing TNBC cells in vitro. Combined treatment with THZ1 and EGFR CAR T cells exhibited stronger cellular toxicity than THZ1 or EGFR CAR T cells alone (Fig. 5A). To confirm that THZ1 inhibition of immunosuppressive genes was linked to the improved efficacy of CAR T cells, overexpression of CD274 (PD-L1), TNFRSF14 (HVEM), and IDO1 exhibited no significant impact on THZ1–induced cellular toxicity, whereas it attenuated that of EGFR CAR T-cell treatment alone or cotreatment with EGFR CAR T cells and THZ1 (Fig. 5A–C). THZ1 cotreatment also attenuated EGFR CAR T-cell–induced expression of immunosuppressive genes, and no additional effects were observed when CDK7 or STAT1 was knocked down via CRISPR/Cas9 (Fig. 5D–H). Accordingly, THZ1 improved the efficacy of CAR T cells in killing MDA-MB-231 cells, but knockdown of CDK7 or STAT1 did not further enhance THZ1’s effects (Fig. 5I). These data suggest that THZ1 modulation of immunosuppressive genes is, at least partially, responsible for the
THZ1 suppresses the activation of EGFR CAR T-cell–induced enhancer and immunosuppressive genes. A and B, MDA-MB-231 cells incubated with CTL or EGFR CAR T cells were incubated with or without THZ1 (250 nmol/L), JQ1 (100 nmol/L), C646 (20 µmol/L), MG149 (200 µmol/L), KDM5-C70 (5 µmol/L), or CDK9 (5 µmol/L) for 48 (A) or 72 (B) hours. MDA-MB-231 cells were subjected to qRT-PCR (A) or immunoblotting (B). Experiments were repeated three times, and representative data are shown. Statistical significance is shown in Supplementary Table S2. ns, nonsignificant; *, P < 0.05; **, P < 0.01; ***, P < 0.001; unpaired Student t test, two-tailed.

Molecular weight is indicated on the right.

C, MDA-MB-231 cells were incubated with or without EGFR CAR T cells in the presence or absence of THZ1 (250 nmol/L) for 48 hours, followed by RNA-seq analysis.

D and E, Heat map (D) and box plot (E) representation of the expression (FPKM, log2) for genes that were induced by EGFR CAR T cells, but suppressed by THZ1. Box plot shows median (line), top and bottom quartiles (boxes), and lines extending to highest and lowest observations (whiskers). P values are shown at the top of the box plot; paired Student t test, two-tailed. F, The expression (FPKM, log2) of representative immunosuppressive genes from RNA-seq as described in C. G, MDA-MB-231 cells were incubated with or without IFNγ (10 ng/mL) in the presence or absence of THZ1 (250 nmol/L) for 48 hours, followed by RNA-seq analysis. H and I, Heat map (H) and box plot (I) representation of the expression (FPKM, log2) for genes that were induced by IFNγ, but suppressed by THZ1. Box plot shows median (line), top and bottom quartiles (boxes), and lines extending to highest and lowest observations (whiskers). P values are shown at the top of the box plot; paired Student t test, two-tailed.

J, The expression (FPKM, log2) of representative immunosuppressive genes from RNA-seq as described in G.

K–N, MDA-MB-231 cells as described in C or G were subjected to qRT-PCR (K and L) or immunoblotting (M and N). Experiments were repeated three times, and representative data are shown. Statistical significance is shown in Supplementary Table S2. *, P < 0.05; **, P < 0.01; ***, P < 0.001; unpaired Student t test, two-tailed. Molecular weight is indicated on the right.
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Figure A: siCTL + – – –
siCDK7 + – – –
CTL T + + + +
CAR-T + + + +

Expression of genes (log2)

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Figure B: siiCTL + – – –
siCDK7 + – – –
PBS + – – –
IFNγ + – – –

Expression of genes (log2)

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Figure E: Pol II ChIP-seq average gene
(genes induced by CAR-T, but suppressed by THZ1, n=1,585)

Normalized tag density

-5.0 Kbp TSS 0 5.0 Kbp

Figure F: Pol II Ser5pho ChIP-seq average gene
(genes induced by CAR-T, but suppressed by THZ1, n=1,585)

Normalized tag density

-5.0 Kbp TSS 0 5.0 Kbp

Figure G: Normalized Pol II tag density
Centered on H3K27Ac enhancer sites nearby CAR-T-cell-induced genes (bp)

Figure H: Normalized Pol II tag density
Centered on H3K27Ac enhancer sites nearby CAR-T-cell-induced genes (bp)

Figure I: Normalized Pol II Ser5pho tag density
Centered on H3K27Ac enhancer sites nearby CAR-T-cell-induced genes (bp)

Figure J: Normalized Pol II Ser5pho tag density
Centered on H3K27Ac enhancer sites nearby CAR-T-cell-induced genes (bp)

Figure K: chr5:131,684,408-131,958,357

Figure L: chr1:110,432,849-110,494,000

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improved efficacy of THZ1. It should be noted that THZ1 itself exhibited minor, yet significant, cellular toxicity toward CAR T cells (Supplementary Fig. S4).

Combination treatment with EGFR CAR T cells and THZ1 suppresses TNBC in mice

The improved efficacy of THZ1 prompted us to examine whether cotreatment with THZ1 alleviated EGFR CAR T-cell–associated immune resistance in mice. SCID mice were subcutaneously implanted with MDA-MB-231 cells, and tumor growth/metastasis was monitored via bioluminescence imaging and caliper-based sizing (Fig. 6A–D; Supplementary Fig. SSA–SSD). Some mice in the control group survived for 45 days (Fig. 6B and E). In the THZ1–treated group, two thirds of the mice exhibited resistance (i.e., the tumor started to grow at a similar rate as the control group) and did survive until the end of the observation, and the remaining one third were alive with tumors (Fig. 6B and E). In the EGFR CAR T–cell–treated group, one third of the mice exhibited resistance to CAR T-cell treatment at approximately 30 days, one third were alive with tumors at the end of the observation, and the remaining one third showed no sign of tumors after treatment, which was consistent with our previous report (ref. 6; Fig. 6B and E). However, mice treated with a combination of THZ1 and EGFR CAR T cells showed no signs of tumors as early as 30 days after treatment. Even after 2 months of treatment withdrawal, when the observation ended, mice showed no sign of tumor recurrence, indicating that combination treatment eradicated MDA-MB-231 cell–originated tumors in mice (Fig. 6B and E). We also rechallenged surviving mice with 10-fold more tumor cells, and prolonged survival was observed, indicating that these mice attained long-term CAR T-cell memory responses (Supplementary Fig. S5E).

The effects of EGFR CAR T cells and THZ1 on tumor growth and metastasis were also evaluated by IHC staining (Fig. 6F–H). Ki67 and EGFR positively stained sections were used to define tumor regions, which were also used to identify and quantify tumor metastases in the tissues (Fig. 6F and G). For EGFR CAR T–cell–treated or THZ1–treated group, tumors exhibiting resistance were selected for staining. Ki67 expression in the primary tumors in the EGFR CAR T–cell–treated or THZ1–treated group was similar to that in the control group (Fig. 6F). The propensity of TNBC for visceral metastasis is higher to the lung than to the lymph nodes, bone, or liver. Consistently, Ki67 staining revealed lung metastasis and fewer liver metastases in the control group, which were abolished in mice cotreated with EGFR CAR T cells and THZ1 (Fig. 6F). EGFR staining showed strong signals (more than 50% cells were EGFR-positive) in primary tumors and lungs but relatively weaker signals (less than 20% cells were EGFR-positive) in the liver of the control group, which was consistent with Ki67 staining (Fig. 6G). EGFR staining was abolished when sections were cotreated with EGFR CAR T cells and THZ1 (Fig. 6G). We also performed CD8 staining to track the infiltration of CAR T cells. As expected, no CD8 staining was observed in both primary tumors and lungs in the control or THZ1–treated group, whereas it was evident in the EGFR CAR T-cell–treated group (Fig. 6H). Combination treatment with EGFR CAR T cells and THZ1 led to the eradication of tumor and tumor metastasis in both lung and liver, and therefore no CD8 staining (i.e., no residual T cells) was observed, further strengthening the specificity of EGFR CAR T cells towards TNBC cells (Fig. 6H). No notable tissue damage was observed in the mice treated with both EGFR CAR T cells and THZ1 (Supplementary Fig. SSF and SSG).

Strong staining for representative immunosuppressive proteins was observed in the primary tumor as well as in the lung and, to a lesser extent, in the liver of EGFR CAR T–cell–resistant tumors, which was significantly attenuated by cotreatment with THZ1 (Supplementary Fig. SSH–SSK). All IHC staining was quantified (Supplementary Fig. S5L–SSR).

The effect of the combination treatment was further tested in an EGFR CAR T–cell–resistant MDA-MB-231 cell–derived xenograft model (Fig. 7A). EGFR CAR T cells exhibited no significant effects on tumor growth after approximately 14 days of treatment, suggesting that the resistant model was successful. However, THZ1 cotreatment sensitized the resistant tumor cells to CAR T-cell treatment (Fig. 7B–D), which was associated with the infiltration of T cells, represented by the expression of CD3 and CD8 (Fig. 7E–G). We observed a rapid increase in CAR gene copies after treatment for 3 days (>10,000 copies/μg gDNA), which decreased sharply in the EGFR CAR T–cell–treated group but decreased gradually and remained at a high level in the EGFR CAR T cells and THZ1 cotreated group even after 20 days (>5,000 copies/μg gDNA; Fig. 7H). EGFR CAR T-cell treatment led to increased expression of immunosuppressive genes, which was abolished in the presence of THZ1 (Fig. 7I; Supplementary Fig. S5F). Similar observations were made for IL6 and IDO1 in the serum (Fig. 7J).

We also tested the effect of EGFR CAR T cells and THZ1 cotreatment on TNBC in a PDX model (Supplementary Fig. S7A and S7B). Cotreatment with THZ1 and EGFR CAR T cells displayed the most dramatic effects in suppressing the growth of the PDX (Supplementary Fig. S7C–S7E). The induction of immunosuppressive genes by EGFR CAR T–cell treatment was significantly attenuated when cotreated with THZ1 (Supplementary Fig. S7F).

Finally, the effect of EGFR CAR T cells and THZ1 cotreatment on TNBC was evaluated using an immunocompetent model. We first constructed a retroviral vector encoding EGFR–targeted CAR designed for mice (EGFR mCAR; Supplementary Fig. S8A), which had high expression in HEK293T cells after transfection (92.2%; Supplementary Fig. S8B). Mouse T cells were infected with retroviruses encoding EGFR mCAR to generate EGFR mCAR T cells (Supplementary Fig. S8C). EGFR mCAR T cells killed murine TNBC EMT6 cells in a dose- and time-dependent manner (Supplementary Fig. S8D). THZ1 enhanced the efficacy of EGFR mCAR T cells in killing EMT6 cells in vitro (Supplementary Fig. S8E). The EGFR mCAR T–cell–induced expression of immunosuppressive genes was attenuated by THZ1.
Figure 5.
THZ1 modulates the expression of immunosuppressive genes to improve the efficacy of EGFR CAR T cells in vitro. A, MDA-MB-231 cells stably expressing control vector (PCDH-CTL 231), PD-L1 (PCDH-PD-L1 231), IDO1 (PCDH-IDO1 231), or HVEM (PCDH-HVEM 231) were incubated as indicated, followed by cytotoxicity assay. Statistical significance for each condition was performed between control and stable cells. Mean ± SEM; ns, nonsignificant; *, P < 0.05; **, P < 0.01; unpaired Student’s t-test, two-tailed. B and C, The cells described above were subjected to qRT-PCR (B) or immunoblotting (C). Experiments were repeated three times, and representative data are shown. Mean ± SEM; ***, P < 0.001; unpaired Student’s t-test, two-tailed. Molecular weight is indicated on the right. D and I, MDA-MB-231 cells stably expressing control sgRNA (sgCTL), sgRNA specific against STAT1 (sgSTAT1), or CDK7 (sgCDK7) were incubated as indicated, followed by qRT-PCR (D) or cytotoxicity assay (I). Experiments were repeated three times, and representative data are shown. Mean ± SEM; ns, nonsignificant; **, P < 0.01; ***, P < 0.001; unpaired Student’s t-test, two-tailed. E–H, The knockdown efficiency of STAT1 (E and F) and CDK7 (G and H) was examined by qRT-PCR (E and G) and immunoblotting (F and H). Mean ± SEM; ***, P < 0.001; unpaired Student’s t-test, two-tailed. Molecular weight is indicated on the right.
Combination treatment with EGFR CAR T cells and THZ1 cures TNBC in a MDA-MB-231 cell–derived xenograft model in mice. A, Six-week-old female SCID mice were injected s.c. with MDA-MB-231 cells stably expressing a luciferase reporter (MDA-MB-231-fluc, 5.0 × 10^5 cells/mouse), and randomized for treatment 5 days later (day 0; 3 mice/group). Mice were treated with CTL T or EGFR CAR T cells (5 × 10^6 cells/dose, i.v. injection) in the presence or absence of THZ1 (10 mg/kg/dose, i.p. injection) following the protocol as depicted. B, The growth of tumors was monitored by bioluminescence imaging. C and D, The tumor growth curves based on bioluminescence (C) and tumor volumes (D) are shown. Mean ± SEM; *, P < 0.05; **, P < 0.01; ***, P < 0.001; two-way ANOVA with Dunnett multiple comparisons test. E, The survival curves of mice in B. Significance was calculated using log-rank (Mantel–Cox) test; *, P < 0.05; **, P < 0.01; ***, P < 0.001. F–H, Sections of primary tumors, livers, and lungs from mice sacrificed at day 45 as described in B were subjected to IHC staining. Representative positive staining is indicated by red arrows. Scale bar, 50 μm. Boxed area shown in the zoomed image, scale bar, 50 μm.
Figure 7.

Cotreatment with THZ1 resensitizes EGFR CAR T-cell-resistant tumor cells to CAR T-cell treatment. **A**, Six-week-old female SCID mice were transplanted s.c. with CAR T-cell-resistant tumor cells (2 × 10⁵ cells/mouse) and randomized for treatment 2 weeks later (day 0; 5 mice/group). Mice were treated with CTL T or EGFR CAR T cells (5 × 10⁶ cells/dose; i.v. injection) in the presence or absence of THZ1 (10 mg/kg/dose, i.p. injection) following the protocol as depicted. **B**, The photographs of the excised tumors at the end of experiment (day 35). **C**, The growth curves of tumors as described in **A**. Mean ± SEM; ns, nonsignificant; *, P < 0.05; **, P < 0.01; ***, P < 0.001; two-way ANOVA with Dunnett multiple comparisons test. **D**, The average of tumor volume. Mean ± SEM; ns, nonsignificant; *, P < 0.05; **, P < 0.01; ***, P < 0.001; one-way ANOVA with Holm–Sidak multiple comparisons test. **E**, The expression of human CD3 and CD8 was examined by qRT-PCR. Experiments were repeated three times, and representative data are shown. Mean ± SEM; ***, P < 0.001; unpaired Student t-test, two-tailed. **F**, IHC staining for CD8 in excised tumors. Red arrows indicate representative positive staining. Scale bar, 50 μm. Boxed area shown in the zoomed image, scale bar, 50 μm. **G**, Quantification of CD8-positive staining over three mice in each group as shown in **F**. Mean ± SEM; ***, P < 0.001; unpaired Student t-test, two-tailed. **H**, The gDNA of PBMCs was analyzed to measure the copy number of CAR gene. **I**, The expression of immunosuppressive genes was examined in excised tumors. Statistical significance is shown in Supplementary Table S2. *, P < 0.05; **, P < 0.01; ***, P < 0.001; unpaired Student t-test, two-tailed. **J**, IL6 and IDO1 in serum were determined by ELISA. Mean ± SEM; ***, P < 0.001; unpaired Student t-test, two-tailed.
(Supplementary Fig. S8F). Similar to what we observed in xenografts from human TNBC cell lines or patients, EGFR mCAR T cells and THZ1 cotreatment exhibited the most significant effects on EMT-derived allografts (Supplementary Fig. S8G–S8I). The expression of immunosuppressive genes was attenuated by THZ1 (Supplementary Fig. S8K).

Discussion

To overcome EGFR CAR T-cell–associated immune resistance, we sought to determine the molecular mechanisms by performing transcriptomic analysis in TNBC tumor cells from EGFR CAR T-cell–resistant xenografts, which revealed that a large cohort of immuno-suppressive genes was strongly induced. These genes were found to be associated with EGFR CAR T-cell–induced enhancers and were especially sensitive to THZ1, a CDK7 inhibitor. Combination therapy with THZ1 and EGFR CAR T cells suppressed immune resistance, tumor growth, and metastasis in TNBC xenograft and allograft models in mice (Supplementary Fig. S9).

Small-molecule inhibitors or mAbs targeting EGFR are being evaluated in clinical trials for TNBC (35). We have shown that EGFR CAR T cells exhibit potent and specific cytotoxicity against TNBC in cultured cells and mice (6). Like many other immuno-therapies, tumors soon acquire resistance after treatment, which limits their application in patients with cancer. Multiple factors contribute to the acquired resistance, such as the emergence of tumor-mediated immunosuppressive mechanisms (12). Through transcriptomic analysis, we found that a large cohort of immuno-suppressive molecules was induced after EGFR CAR T-cell treatment in TNBC cells, which were primarily regulated by IFNγ signaling (6). As we reported previously, the cytotoxicity of EGFR CAR T cells was also mediated through IFNγ signaling. IFNγ signals are early immune responses in T-cell activation and differentiation, which predict clinical responses to immune checkpoint blockade therapy (36–38). However, in certain circumstances, such as chronic exposure, IFNγ can induce tumor progression and/or drug resistance (39–41). Therefore, IFNγ serves as a double-edged sword: killer and protector (39).

The observation that a large cohort of immunosuppressive genes was induced after EGFR CAR T-cell treatment prompted us to find a way to systematically interfere with this cohort rather than focus on individual genes, aiming to reduce the associated immune resistance. EGFR CAR T-cell–induced immunosuppressive genes were found to be associated with enhancers. By testing several inhibitors targeting known functional players on active enhancers, we found that THZ1 displayed the best effect on attenuating the expression of EGFR CAR T-cell–induced immunosuppressive genes. Although the link between the set of interferon signaling genes and the improved efficacy of THZ1 remains unclear, our data suggest that the effects of THZ1 on the set of IFNγ signaling genes account for, at least partially, the improved efficacy of EGFR CAR T cells by THZ1. It has been shown that an “Achilles cluster” of superenhancer-associated, TNBC-specific genes is especially sensitive to CDK7 inhibition/THZ1 (29), which was also seen in our RNA-seq analysis. The inhibitory effects of THZ1 on these genes may also contribute to the improved efficacy of THZ1. Although we demonstrated that CDK7 is, at least partially, responsible for the improved efficacy of THZ1, it is worth noting that THZ1 is a covalent inhibitor that also targets CDK12/13 (28, 42). CDK7 inhibition using YKL-5-124 activates IFNγ and TNFα signaling in small-cell lung cancer (43). Unlike THZ1, cells incubated with YKL-5-124 display little change in RNA Pol II phosphorylation (44). Here, EGFR CAR T-cell–induced interferon signaling genes were subjected to the regulation of Pol II phosphorylation, and THZ1 inhibited CDK7–mediated Pol II phosphorylation to suppress these genes. The different working mechanisms might be, at least in part, why THZ1 and YKL-5-124 exhibit different effects on gene expression. The IFN signaling genes are regulated by cell-type–specific enhancers, which might also account for the observed differences.

In summary, we discovered that tumor cells acquire immune resistance to CAR T-cell treatment due to CAR T-cell–activated enhancer and–cognate immunosuppressive genes, and we identified an effective way to disrupt the transcription of these genes to reduce immune resistance. Unlike the usual “divide-and-conquer,” we took advantage of a “unite-and-conquer” strategy to suppress the expression of a cohort of genes by using inhibitors targeting a transcriptional modulator. Our strategy may apply to other cancer immunotherapies. This strategy may also be effective in treating other difficult-to-treat cancers with EGFR overexpression. Due to the critical role of STAT in the IFNγ signaling pathway, combination therapy with EGFR CAR T cells and inhibitors of the JAK/STAT signaling pathway might also be effective in overcoming the acquired immune resistance associated with EGFR CAR T cells. Combining inhibitors targeting some of the critical immunosuppressive genes induced by EGFR CAR T-cell treatment, such as PD-L1/2, with EGFR CAR T cells may also be promising. Future investigations will be required to determine whether these therapeutic strategies can be translated into clinical settings.

Authors’ Disclosures

No disclosures were reported.

Authors’ Contributions


Acknowledgments

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Targeting Triple-Negative Breast Cancer with Combination Therapy of EGFR CAR T Cells and CDK7 Inhibition


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