

Resistance of breast cancer stem cells to TROP2-targeted CAR-T therapy via the NF- κ B signaling pathway

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Abstract

Background

Breast cancer is a prevalent female malignancy with poor treatment outcomes, and cancer stem cells (CSCs) mainly cause tumor relapse and drug resistance. This study aimed to elucidate the molecular mechanisms underlying the resistance of breast CSCs (BCSCs) to trophoblast cell surface antigen 2 (TROP2)-targeted chimeric antigen receptor T (CAR-T) therapy, with a specific focus on the regulatory function of the NF- κ B signaling pathway.

Methods and Results

TROP2-targeted CAR-T efficiently eliminated TROP2-positive MCF7 cells and MDA-MB-231 cells. *In vivo* experiments further verified the antitumor efficacy of TROP2-targeted CAR-T in suppressing tumor growth in mouse models. Notably, TROP2-targeted CAR-T treatment induced significant CSC enrichment *in vitro* and *in vivo*, suggesting that BCSCs are intrinsically resistant to CAR-T therapy. Mechanistically, mass spectrometry analysis revealed marked activation of the NF- κ B signaling pathway in CSCs. Consistent with these findings, subsequent validation confirmed that TROP2-targeted CAR-T treatment markedly activated the phosph-p65, phosph-p38, and phosph-pERK signaling pathways both *in vitro* and *in vivo*.

Conclusions

Our findings demonstrate that BCSCs mediate TROP2-targeted CAR-T resistance via NF- κ B pathway activation. Targeting this signaling axis may enhance the efficacy of CAR-T therapy in breast cancer. Furthermore, combinatorial strategies deserve further investigation to overcome such resistance and improve patient outcomes.

Introduction

Breast cancer remains one of the most prevalent malignancies affecting women globally, presenting significant therapeutic challenges due to its complex biology and the presence of breast cancer stem cells (BCSCs)^[1–3]. CSCs play important roles in tumor initiation and progression, as well as resistance to conventional therapies. Although extensive studies have investigated the chemoresistance mechanisms of CSCs, their responsiveness to novel immunotherapies, including chimeric antigen receptor T (CAR-T) therapy, remains poorly understood. Accumulating evidence has validated the remarkable efficacy of CAR-T-cell therapy in a spectrum of hematological malignancies^[4–7]. However, its therapeutic efficacy remains limited in solid tumors such as breast cancer, underscoring the urgent need to elucidate the intrinsic resistance mechanisms involved.

Current evidence suggests that CSCs are major drivers of breast cancer resistance to CAR-T-cell therapy through the activation of multiple oncogenic signaling pathways^[8, 9]. Among these signaling axes, the NF-κB and MAPK cascades have been recognized as particularly pivotal regulators^[10]. Mechanistically, both pathways orchestrate a broad spectrum of core cellular biological processes, including malignant cell survival, uncontrolled proliferation, and tumor-associated inflammatory responses. These downstream cellular events collectively create an immunosuppressive tumor microenvironment (TME) and ultimately impair the antitumor activity and overall therapeutic efficacy of CAR-T-based immunotherapeutic interventions. Although the clinical outcomes of CAR-T therapy in a variety of hematological and solid malignancies are remarkable and encouraging^[6, 11–14], the precise biological interplay between BCSCs and CAR-T cells, as well as the upstream regulatory networks driving therapeutic resistance, remain poorly understood.

Tumor-associated antigen expression critically affects CAR-T therapy efficacy in patients with malignancies^[13, 15–17]. Trophoblast cell surface antigen 2 (TROP2) is a promising CAR-T target for breast cancer, especially triple-negative breast cancer (TNBC)^[18, 19], owing to its aberrant overexpression; preclinical studies have confirmed that TROP2 overexpression sensitizes breast cancer cells to TROP2-targeted CAR-T cytotoxicity via specific binding and activation of antitumor cascades, including apoptosis and lysis^[18]. However, the heterogeneous TME of breast cancer—which consists of varied tumor, stromal, and immune cells and extracellular matrix—leads to prominent heterogeneity in TROP2 expression. This heterogeneity severely impedes the clinical translation, efficacy optimization and broad application of TROP2-targeted CAR-T therapy; therefore, resolving this bottleneck is imperative to improve therapeutic outcomes.

To address these clinical challenges, this study aimed to elucidate the mechanism by which BCSCs mediate resistance to TROP2-targeted CAR-T therapy via the NF-κB signaling pathway. We integrated *in vitro* assays of breast cancer cell lines with differential TROP2 expression, *in vivo* mouse models, mass spectrometry and RNA sequencing to characterize biological and molecular changes in BCSCs following TROP2-CAR-T treatment. Multiomics analysis was further employed to identify key activated signaling cascades and druggable targets for improving CAR-T efficacy in breast cancer. Elucidating BCSC-driven CAR-T resistance mediated by the NF-κB pathway is essential for optimizing clinical outcomes. This work aims to develop novel combinatorial strategies to overcome the limitations of current CAR-T therapy for breast cancer, which will advance the mechanistic understanding of immune resistance in breast cancer and lay a foundation for the development of more effective immunotherapeutic regimens.

Method

Cell culture and tumor sphere formation assay

Human breast cancer cell lines (MCF7 and MDA-MB-231 cells) were purchased from American Type Culture Collection (ATCC) and maintained in DMEM (Thermo Fisher Scientific) supplemented with 10%

heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, and 1% penicillin/streptomycin (all purchased from Thermo Fisher Scientific); all the cell lines were cultured at 37°C in a humidified chamber with 5% CO₂. A SOX2 reporter cell line was constructed by infecting parental MCF7 and MDA-MB-231 cells with lentiviral supernatants containing a SOX2 reporter and selecting them with puromycin. MDA-MB-231 SOX2 reporter cell lines were also infected with lentiviral supernatants containing IRES-GFP for *in vivo* experiments.

For the tumor sphere culture assay, cells were grown in ultralow attachment plates (Corning, NY, USA) with sphere medium supplemented daily with growth factors [20 ng/mL fibroblast growth factor (FGF), 20 ng/mL EGF; Sigma–Aldrich] as described previously^[20]. One hundred cells were seeded into 200 µl of medium and propagated in 96-well ultralow attachment plates (Corning, NY, USA) at 37°C and 5% CO₂. Spheres were counted between days 5 and 9. To investigate serial sphere formation, the spheres were washed with PBS and dissociated into single cells by trypsinization.

TROP2-CAR construction

The sequences of the Trop2 antibodies were derived from the ADC drug (sacituzumab govitecan). The variable region sequences of the heavy (VH) and light chain (VL) of this antibody were used to design an scFv with the sequence VH-(G4S)₃ Linker-VL. The TROP2 CAR containing the scFv from sacituzumab govitecan was designated TROP2 scFv. From the 5'-end to the 3'-end, the TROP2 CAR was composed of the human CD8a signal peptide sequence, TROP2 scFv, hinge and TM regions of the human CD8a molecule, CD28 intracellular domain sequence (CD28-ICD), and CD3ζ intracellular domain sequence (CD3ζ-ICD). DNA encoding the CARs was codon optimized and synthesized by SYN BIO (Jiangsu, China) with appropriate restriction sites.

Lentivirus production and transduction

High-titer replication-incompetent lentiviruses were produced and concentrated as described previously^[21]. In brief, HEK293T cells were transfected with the target plasmid and the helper plasmids pSPAX and pMD2.G using Lipofectamine 2000 (Invitrogen). The viral supernatant was harvested at 48 and 72 h after transfection and concentrated using Lenti-X Concentrator (Clontech) in accordance with the manufacturer's instructions.

Lentiviruses carrying CAR sequences and SOX2 enhancer reporter constructs were designed, produced, and used for transduction as previously reported^[22].

CAR-T-cell production

Human PBMCs were isolated by density gradient centrifugation over Ficoll-Paque (GE Life). T cells were isolated from human PBMCs by CD⁴/CD⁸ microbeads and then activated with TransAct (Miltenyi Biotec)

at a 1:100 ratio for 48 h, followed by transduction with lentiviruses, and vectofusion-1 was added to increase the infection efficacy. Infected T cells were then maintained in OptiViro T-cell serum-free basal culture (ExCell) supplemented with recombinant human IL-2 (300 U/ml). Fresh medium containing cytokines was replenished every other day to maintain the T-cell concentration at 0.5×10^6 cells/ml. CAR-T cells were propagated for 9 days in total before use in functional assays. To track T-cell numbers over time, viable cells were counted using trypan blue.

Flow cytometry

TROP2 expression in MCF7 and MDA-MB-231 cells was detected with an anti-TROP2-APC antibody (REA916, Mittenyl). The cells were stained with a Viability Kit (Fixable Viability Stain 780, BD Biosciences) to exclude dead cells from the analysis. Flow cytometry data were acquired with a BD LSRFortessa (BD Biosciences) using DIVA software according to the manufacturer's instructions.

In vitro killing assays

For tumor cell killing assays, MCF7 or MDA-MB-231 cells (1×10^6 cells/well) were cocultured with MOCK- or TROP2-targeted CAR-T cells at varying effector-to-target ratios in triplicate wells of white 6-well plates. Target cell viability was analyzed at different time points.

Immunoblotting

Cells were disrupted in 1× Lysis Buffer (Thermo Fisher Scientific) supplemented with Protease/Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific). The proteins were stained with the following primary antibodies: anti-phospho-p65 (82335, Proteintech), anti-p65 (10745, Proteintech), anti-phospho-p38 MAPK (28796, Proteintech), anti-phospho-ERK1/2 (28733, Proteintech), mouse anti-actin (3700S, Cell Signaling Technology) and anti-GAPDH (5174P, Cell Signaling Technology). Detection was carried out using ECL Prime Western Blotting Detection Reagent (Thermo Fisher Scientific).

Immunohistochemistry

The tumor tissues were fixed with formalin and embedded in paraffin. Then, 5-mm-thick sections were deparaffinized with xylene and rehydrated in decreasing concentrations of ethanol. After heat-induced antigen retrieval, the slides were blocked with 3% BSA and stained with anti-hCD3 (ptm-5013, Jingjie), anti-hCD8 (ab189926, Abcam), Td Tomato (orb182397, Biorbyt), anti-hPDL1 (ab237726, Abcam), anti-phospho-p65 (82335, Proteintech) and anti-BCL2 (abs115024, Absin) antibodies in blocking solution overnight at 4°C. Slides were visualized with digital slide scanners (PANNORAMIC SCAN II).

Mass spectrometry

The SOX2 reporter MCF7 cells were sorted into RFP⁺ and RFP⁻ groups. Two groups of samples were selected, and each group was represented by three biological replicates. All six samples were processed by data independent acquisition (DIA) individually to assess the proteome differences. The peptides were analyzed by online nanoflow liquid chromatography–tandem mass spectrometry performed on an EASY-nanoLC 1200 system (Thermo Fisher Scientific) connected to an orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific). MS1 and MS2 data were acquired, and sample acquisition occurred in random order. The iRT kit (Ki3002, Biognosys AG, Switzerland) was added to all of the samples to calibrate the retention times of the extracted peptide peaks. Statistical analysis of the DIA dataset was performed using Spectronaut 14 (Biognosys AG, Switzerland), including data normalization and relative protein quantification. After Welch's ANOVA test, differentially expressed proteins were filtered if their |fold change| was > 1.3.

mRNA sequencing

MCF7 cells were treated with MOCK- or TROP2-targeted CAR-T cells at a 1:1 E:T ratio for 6 days. For mRNA sequencing, T cells were removed from the treated cells. The indexed cDNA sequencing libraries were prepared from the RNA samples using a Hieff NGS® Ultima Dual-mode mRNA Library Prep Kit. Quality control was performed at every step, and the libraries were quantified using an Agilent 2100 Bioanalyzer. Indexed libraries were prepared as equimolar pools and generated a minimum of 30 million paired-end reads per sample library. The raw sequence data were demultiplexed and converted to fastq files, and adaptor and low-quality sequences were trimmed.

Zebrafish xenografts

Sorted MCF7 and MDA-MB-231 RFP⁺/RFP⁻ cells were separately labeled with the fluorescent CellTracker™ CM-Dil (Life Technologies), a lipophilic fluorescent tracking dye, according to the manufacturer's instructions. Tg (flk1:eGFP) zebrafish were maintained, collected, grown and staged in E3 medium at 28.5°C according to standard protocols. For the xenotransplantation experiments, zebrafish embryos were anesthetized in 0.4% tricaine (Sigma) at 48 h postfertilization (hpf), and 50 or 100 MCF7/MDA-MB-231 cells were microinjected into the vessel-free area of the yolk sac. The embryos were incubated for 1 h at 28.5–29°C for recovery, and cell transfer was verified by fluorescence microscopy. Fish harboring red cells were incubated at 35°C essentially as described previously. On assay day 5, the embryos were screened microscopically for tumor cell engraftment using a Zeiss LSM 710 confocal microscope, and the number of tumor-bearing fish was quantified.

In vivo tumor models

All animal experiments were conducted under a protocol approved by the Institutional Animal Care and Use Committee of the Shanghai University of Medicine and Health Sciences. Six-week-old NOD scid gamma (NSG) (nonobese diabetic/severe combined immunodeficiency/interleukin-2 receptor γ -chain null) mice were obtained from Gempharmatech Co., Ltd. (Suzhou, China). A tumor xenograft model was established by subcutaneous (s.c.) inoculation with 10⁶ MDA-MB-231 cells suspended in 100 μ l of PBS.

After 2 weeks, when the tumors were consistently palpable (50–100 mm³), the mice were randomized into two groups (three to five mice per group) and intravenously (i.v.) injected with 5×10^6 MOCK or TROP2-targeted CAR-T cells suspended in 100 μ l of PBS. After 2 weeks, the mice were euthanized by isoflurane, and blood and tumors were harvested for analysis. Tumor area (A) was analyzed using ImageJ software.

Statistics

Statistical analyses were performed using SPSS version 22 (IBM, Ehningen, Germany), R Studio 3.5.0, and GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). Unless otherwise indicated, data from ≥ 3 independent biological triplicates were analyzed using Student's t test or one-way analysis of variance (ANOVA) * $P \leq 0.05$, ** $P \leq 0.001$, and *** $P \leq 0.0001$. The error bars indicate the standard deviations (SDs).

Results

BCSCs exhibit resistance to TROP2-targeted CAR-T-cell therapy

In this study, we first investigated the association between TROP2 expression and prognosis in patients with TNBC and then verified whether BCSCs mediate resistance to TROP2-targeted CAR-T-cell therapy. Kaplan–Meier survival analysis revealed a significant difference in overall survival between patients with TNBC with high and low TROP2 expression (Fig. 1A), indicating that TROP2 is correlated with TNBC prognosis. Subsequently, second-generation CAR constructs, including TROP2-targeted CAR and control MOCK CAR, which both contain the CD28 costimulatory domain to ensure CAR-T-cell activation, were constructed (Fig. 1B). Short-term cytotoxicity assays revealed that TROP2-targeted CAR-T cells exerted a significant killing effect on MCF7 and MDA-MB-231 cells at E:T ratios of 10:1, 5:1, and 1:1 for 24 and 48 h (Fig. 1C–D). However, long-term coculture assays (2, 4, and 6 days) at E:T ratios of 5:1 and 1:1 revealed marked tumor therapeutic resistance (Fig. 1E–F). Flow cytometry revealed a dynamic increase in the number of RFP⁺ BCSCs, which significantly increased in a time- and E:T ratio-dependent manner following CAR-T treatment (Fig. 1G–H). Quantitative analysis at 6 days postculture confirmed significantly increased proportions of TROP2⁺ and RFP⁺ cell subsets (Fig. 1K–L). Collectively, these findings confirm that TROP2-targeted CAR-T cells exhibit short-term therapeutic efficacy while inducing long-term tumor resistance and that BCSCs serve as the key cell population that mediates this resistance.

Characteristics and underlying signaling mechanism of RFP⁺ cells

To systematically characterize the biological properties of RFP⁺ cells, sphere-forming assays were performed using FACS-sorted RFP⁺ and RFP[−] subpopulations isolated from the MCF7 and MDA-MB-231

breast cancer cell lines, respectively. To further evaluate the self-renewal capacity, which is a key hallmark of BCSCs, serial replating assays were conducted: primary spheres formed in the initial culture were collected, gently dissociated into single-cell suspensions, and then replated at the same density as the primary culture. The results demonstrated that compared with their corresponding RFP⁻ counterparts, RFP⁺ MCF7 and MDA-MB-231 cells exhibited significantly greater sphere-forming efficiency, indicating the enhanced self-renewal potential of RFP⁺ cells (Fig. 2A–B). Consistently, *in vivo* tumorigenicity assays were carried out by implanting FACS-isolated RFP⁺ and RFP⁻ MCF7 and MDA-MB-231 cells into zebrafish models. Quantitative analysis of tumor formation revealed that compared with RFP⁻ cells, RFP⁺ cells displayed markedly greater tumorigenic capacity, with a higher tumor formation rate and larger tumor size,, further confirming the BCSC-like properties of RFP⁺ cells (Fig. 2C–D).

To explore the underlying molecular signaling mechanisms mediating the CSC-like characteristics of RFP⁺ cells, we subjected FACS-sorted RFP⁺ and RFP⁻ MCF7 cells to mass spectrometry analysis to identify differentially expressed proteins (DEPs) between the two groups. Based on the DEPs, functional enrichment analysis was performed, and the top 20 significantly enriched signaling pathways, including KEGG pathways, were identified (Fig. 2E–F). These results provide direct evidence that the NF- κ B and cellular senescence signaling pathways are specifically activated in RFP⁺ CSCs, which may serve as key regulatory pathways underlying their enhanced self-renewal and tumorigenic capacities.

TROP2-targeted CAR-T treatment affects immune system processes in breast cancer cells in vitro

To compare the transcriptional profiles of tumor cells treated with TROP2-targeted CAR-T cells versus MOCK CAR-T cells, RNA sequencing was performed on samples from both groups. Enrichment analysis of the 2897 identified differentially expressed genes (DEGs) (FC > 1.5, P < 0.05) revealed the top 20 enriched pathways from the general enrichment analysis (Fig. 3A), which revealed distinct pathway enrichment profiles between the two groups. These pathways were predominantly associated with immune functions such as T-cell differentiation and immune cell proliferation. Additionally, KEGG enrichment analysis confirmed the top 20 enriched KEGG pathways (Fig. 3B), including the JAK–STAT, TNF and NF- κ B signaling pathways, suggesting that TROP2-targeted CAR-T cells may offer functional advantages, such as modulating these critical signaling pathways. Furthermore, GSEA was conducted to verify the enrichment of specific functional gene sets involved in the activation of the immune response, the cellular response to cytokine stimulus, regulation of the defense response, regulation of the apoptotic signaling pathway, regulation of cell development, and regulation of cell adhesion (Fig. 3C–H). Collectively, these results indicate that TROP2-targeted CAR-T cells kill breast cancer cells by activating immune signaling pathways, thereby providing a molecular basis for their superior antitumor performance. In addition, TROP2-targeted CAR-T-cell treatment can activate NF- κ B-related pathways in some tumor cells, which may exert a protective effect on these cells.

Trop2-specific CAR-T-cell treatment affects the MAPK and NF- κ B pathways in vitro

We next performed GSEA based on the aforementioned RNA-seq data to screen enriched pathways. The results revealed that the MAPK signaling pathway, NF- κ B signaling pathway, and PD-L1 expression/PD-1

checkpoint pathway in cancer cells were significantly activated following treatment with TROP2-targeted CAR-T cells (Fig. 4A–C). To further verify the signaling mechanism responsible for the anti-breast cancer effects of TROP2-targeted CAR-T cells, we performed immunoblotting to detect the expression levels of phosphorylated P38 (p-P38), phosphorylated P65 (p-P65), and phosphorylated ERK (p-ERK) in MCF7 and MDA-MB-231 cells after they were cocultured with TROP2-targeted CAR-T cells (Fig. 4D–E). Western blot results revealed that the levels of p-P38, p-P65 and p-ERK were markedly upregulated in MCF7 cells upon coculture with TROP2-targeted CAR-T cells, whereas no such obvious activation was observed in MDA-MB-231 cells *in vitro*. Collectively, these results reveal that TROP2-targeted CAR-T cells regulate the MAPK and NF- κ B signaling cascades in breast cancer cells—particularly BCSCs. Mechanistically, activation of these two pathways endows tumor cells with intrinsic resistance to the cytotoxic effects induced by TROP2-CAR-T cells. Second, MAPK and NF- κ B signaling promotes programmed death-ligand 1 (PD-L1) transcription and expression, which further blunts the tumoricidal efficacy of TROP2-CAR-T cells by inducing immune evasion.

Trop2-targeted CAR-T cells effectively inhibit breast cancer development in vivo

To systematically evaluate the antitumor efficacy of TROP2-CAR-T cells, we first established a stable xenograft tumor model in NCG mice using human TNBC MDA-MB-231 cells. The schematic of the detailed experimental design, including the grouping strategy, cell administration schedule, and detection time points, is shown in Fig. 5A, ensuring the reproducibility and rigor of the experiment. After the treatment was completed, representative images of the xenograft tumors from each group were captured, and quantitative analysis of the tumor areas was performed. The results clearly demonstrated that compared with the control group, the TROP2-CAR-T treatment group exhibited significantly smaller tumor areas (Fig. 5B–C), which directly indicated that TROP2-CAR-T cells effectively inhibited *in vivo* tumor growth in the xenograft model.

To further explore the potential molecular mechanism underlying the observed antitumor effect of TROP2-CAR-T cells, immunofluorescence staining was performed on paraffin-embedded tumor tissue sections using a specific CD8 antibody. This staining was designed to detect the infiltration level of CD8⁺ T cells, a key subset of cytotoxic T lymphocytes that play a crucial role in antitumor immunity. Compared with those in the Mock group, the infiltration of CD8⁺ T cells in the tumor tissues in the TROP2-CAR-T treatment group was clearly increased (Fig. 5D–E). Collectively, these findings confirm that TROP2-CAR-T treatment can effectively suppress xenograft tumor growth in NCG mice and that this antitumor effect may be closely associated with the enhanced infiltration of CD8⁺ T cells in tumor tissues, providing a potential mechanism for the clinical application of TROP2-CAR-T-cell therapy.

Trop2-specific CAR-T-cell treatment activates the NF- κ B pathway in vivo

To elucidate the molecular mechanism through which TROP2-CAR-T therapy regulates tumor progression, we performed a panel of immunostaining assays followed by quantitative analysis. First, we assessed the infiltration of RFP⁺ cells within tumor tissues. The results revealed markedly greater RFP⁺ cell infiltration in the TROP2-CAR-T-cell group than in the Mock group (Fig. 6A–B). Next, to determine

whether TROP2-CAR-T treatment modulates PD-L1 expression, we examined the abundance of PD-L1⁺ cells in tumor tissues. Increased PD-L1⁺ cell abundance was observed in the TROP2-CAR-T group, and quantitative analysis confirmed an extremely significant difference in the proportion of PD-L1⁺ cells between the two groups (Fig. 6C–D). We further examined the regulatory role of TROP2-CAR-T treatment in NF-κB pathway activation by immunohistochemical staining of xenograft tumors using a phosphorylated P65 antibody, a canonical biomarker for NF-κB activation. Compared with that in the Mock group, the proportion of phosphorylated P65⁺ cells in the TROP2-CAR-T group was significantly greater (Fig. 6E–F). Moreover, to evaluate the effect of TROP2-CAR-T treatment on the antiapoptotic capability of tumor cells, we performed immunofluorescence staining for BCL2, a critical antiapoptotic protein. Quantitative analysis verified that the proportion of BCL2⁺ cells was significantly greater in the TROP2-CAR-T group (Fig. 6G–H). Taken together, our findings reveal that NF-κB signaling serves as a core mediator of TROP2 CAR-T therapy resistance in BCSCs. Mechanistically, coordinated MAPK and NF-κB signal transduction increases PD-L1 transcription and surface protein abundance, triggering robust tumor immune evasion and consequently dampening the tumoricidal activity of TROP2-engineered CAR-T cells. Furthermore, persistent NF-κB activation increases the expression of the antiapoptotic gene BCL2, enabling BCSCs to evade CAR-T-dependent cell killing through cell-intrinsic prosurvival signaling.

Discussion

The prevalence of breast cancer, the most commonly diagnosed malignancy among women, remains a significant global health challenge^[23, 24]. One of the critical barriers in effectively treating breast cancer is the presence of BCSCs, which are involved in tumor initiation and progression, as well as the development of resistance to therapies^[25, 26]. CAR-T therapy has achieved remarkable success in the treatment of hematologic malignancies^[27, 28], significantly improving the prognosis of patients with relapsed or refractory blood cancers. However, significant challenges are still encountered in the management of breast cancer^[29–31]. Therefore, further elucidation of the intrinsic molecular mechanisms responsible for CAR-T therapy resistance in breast cancer is imperative.

In our preliminary study, we found that high expression of trophoblast cell surface antigen 2 (TROP2) is intimately associated with poor overall survival in patients with TNBC. TROP2 expression patterns differ across various breast cancer cell lines, and such molecular heterogeneity substantially determines cellular responsiveness to TROP2-targeted CAR-T treatment. Preliminary experimental data verified that TROP2-targeted CAR-T treatment effectively suppressed the growth of breast tumor xenografts. Notably, our study further revealed that the proportion of BCSCs was markedly increased following TROP2-targeted CAR-T intervention. Although considerable efforts have been made to elucidate the mechanisms by which BCSCs confer resistance to conventional chemotherapy, how BCSCs respond to novel therapeutic strategies such as CAR-T-cell therapy remains poorly defined. In this context, the present study systematically investigated the therapeutic efficacy of TROP2-targeted CAR-T cells against breast cancer and the pivotal role of BCSCs in mediating CAR-T resistance. The findings are expected to provide novel insights for optimizing CAR-T therapeutic strategies in breast cancer treatment.

Intriguingly, mass spectrometry revealed significant activation of the NF- κ B signaling pathway in BCSCs. Consistent with the proteomic results, our experimental validation further confirmed notable NF- κ B pathway activation in BCSCs following TROP2-targeted CAR-T intervention, suggesting that this signaling cascade plays a critical role in driving BCSC-mediated immunotherapy resistance. In support of this notion, CAR-T treatment markedly increased the phosphorylation level of p65, a core functional subunit of the NF- κ B pathway. Such activated NF- κ B signaling is capable of endowing BCSCs with enhanced survival capacity, thereby enabling these stem-like cells to evade CAR-T-induced cytotoxicity. These results are in good agreement with those of previous studies, which have established a close association between aberrant NF- κ B activation and tumor survival as well as therapeutic resistance in a broad spectrum of malignancies, including breast cancer^[32, 33]. In addition to NF- κ B, the concurrent activation of parallel signaling pathways such as MAPK further adds complexity to the therapeutic response. Collectively, these findings imply that a combinatorial strategy cotargeting the NF- κ B and MAPK cascades may serve as a promising approach to improve the antitumor efficacy of TROP2-targeted CAR-T therapy in breast cancer.

In terms of gene functionality and cellular behavior, our results demonstrate that the enrichment of BCSCs after TROP2-targeted CAR-T treatment is not solely attributable to low expression of the TROP2 antigen. The presence of TROP2-positive BCSCs alongside TROP2-negative cells suggests a more complex interplay between antigen expression and stemness characteristics that influences therapeutic outcomes. These findings underscore the need for a more in-depth understanding of how cancer stem cells adapt to immunotherapeutic pressures and retain their stem-like properties, potentially through epigenetic changes or the activation of survival pathways such as the NF- κ B and MAPK pathways^[34, 35]. By elucidating these underlying mechanisms, we can better understand the challenges posed by cancer stem cells in treatment resistance and identify new targets for intervention that could improve the overall effectiveness of TROP2-CAR-T-cell therapy.

The implications of our findings extend to the broader context of immune mechanisms in cancer treatment. The activation of the NF- κ B pathway in BCSCs suggests that this signaling cascade could play a pivotal role in shaping the immune landscape of tumors and influencing their responses to both immunotherapy and chemotherapy. For instance, NF- κ B has been shown to regulate the expression of various cytokines and chemokines that can modulate immune cell infiltration and activity within the TME^[36, 37]. This highlights the potential for therapeutic strategies that not only target cancer cells directly but also modulate the immune response, enhancing the effectiveness of CAR-T-cell therapies. Further exploration into the roles of NF- κ B and related pathways in this context could pave the way for innovative combination therapies that exploit the vulnerabilities of cancer stem cells while bolstering antitumor immune responses.

In summary, the results of the present study reveal that BCSCs play a pivotal role in driving resistance to TROP2-targeted CAR-T therapy, principally through the activation of the NF- κ B and MAPK signaling axes. Therefore, targeting these two pathways represents a promising strategy for improving the clinical efficacy of CAR-T therapy for breast cancer. Nevertheless, several limitations of this work should be

acknowledged. First, the lack of clinical sample validation weakens the generalizability of our findings, and the relatively limited sample size may compromise the robustness of the conclusions. In addition, in this study, we did not systematically investigate other potential signaling cascades that may also contribute to BCSC-driven therapy resistance, and additional regulatory mechanisms remain to be identified. Collectively, these findings lay a foundation for the development of novel combinatorial therapeutic strategies to overcome CSC-mediated drug resistance. Future well-powered studies with larger clinical cohorts and multipathway profiling are warranted to fully decipher the complex regulatory network underlying CAR-T resistance in breast cancer.

Declarations

Competing interests

The authors declare no competing interests.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Shanghai University of Medicine and Health Sciences. All animal experiments were performed in accordance with relevant institutional guidelines and regulations, and were approved by the Animal Research Ethics Committee of Shanghai University of Medicine and Health Sciences. All procedures and methods were conducted in compliance with the applicable guidelines and regulations.

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Author contributions

Hui Wang designed the experiments, performed the mouse model experiments, funded the project and drafted the manuscript. Qi Wu generated the cell lines utilized in this study. Yuwei Chen and Weiling Chen assisted and participated in cell line-related experiments. Yintao Li provided experimental resources and revised the manuscript. Chunhong Cui funded the project, conceived and designed the study and drafted the manuscript. All authors have read and approved the final manuscript.

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Data Availability

The raw sequencing data generated in this study will be deposited in [Repository name] upon manuscript acceptance. The corresponding accession number will be provided before publication and included in the final published article. No publicly available or secondary datasets were used in this study.

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Figures

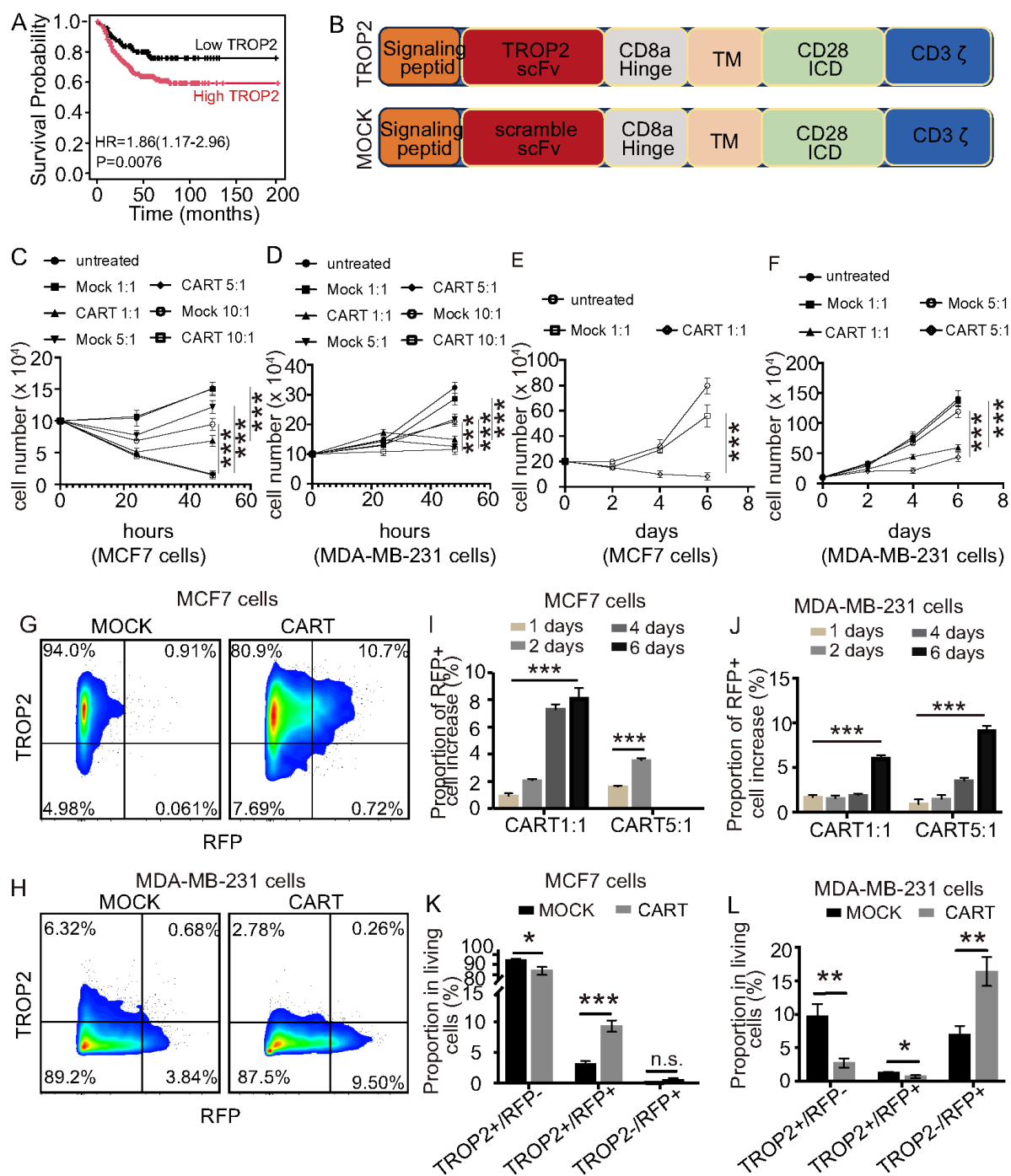


Figure 1

Breast cancer stem cells (BCSCs) exhibit resistance to TROP2-targeted CAR-T cell therapy.

(A) Kaplan–Meier survival analysis of overall survival (OS) in Triple negative Breast Cancer patients stratified into TROP2-high and TROP2-low expression groups.

(B) Schematic structure of the TROP2-targeted CAR and control MOCK CAR. Both are second-generation CAR constructs incorporating the CD28 costimulatory domain.

(C, D) Short-term cytotoxicity assay. MCF7 (C) and MDA-MB-231 (D) cells were co-cultured with MOCK or TROP2-targeted CAR-T cells at E:T ratios of 10:1, 5:1, and 1:1 for 24 and 48 h. Data are shown as mean $\pm$ SEM from three independent experiments. ***P < 0.001. E:T ratio, CAR-T cells to tumor cells.

(E, F) Long-term cytotoxicity assay. MCF7 (E) and MDA-MB-231 (F) cells were co-cultured with MOCK or TROP2-targeted CAR-T cells at E:T ratios of 5:1 and 1:1 for 2, 4, and 6 days. Data are shown as mean $\pm$ SEM with three independent replicates. ***P < 0.001. E:T ratio, CAR-T cells to tumor cells.

(G, H) Schematic workflow of the flow cytometry experimental design. TROP2-targeted CAR-T cells were co-cultured with MCF7 cells at an E:T ratio of 1:1 and MDA-MB-231 cells at an E:T ratio of 5:1 for 6 days. ***P < 0.001. E:T ratio, CAR-T cells to tumor cells. E:T ratio, CAR-T cells to tumor cells.

(I, J) Dynamic changes in the proportion of RFP-positive cells detected by flow cytometry following co-culture of TROP2-targeted CAR-T cells with MCF7 (I) and MDA-MB-231 (J) cells at E:T ratios of 5:1 and 1:1 for 1, 2, 4, and 6 days. The proportion of RFP⁺ cells at day 1 with an E:T ratio of 1:1 was normalized to 1. n = 3 biological replicates; ***P < 0.001. E:T ratio, CAR-T cells to tumor cells.

(K, L) Flow cytometric quantification of TROP2⁺ and RFP⁺ cell populations after 6 days of co-culture. MCF7 cells were treated at an E:T ratio of 1:1 (K), and MDA-MB-231 cells at an E:T ratio of 5:1 (L). n = 3 biological replicates; *P < 0.05, **P < 0.01, ***P < 0.001. E:T ratio, CAR-T cells to tumor cells.

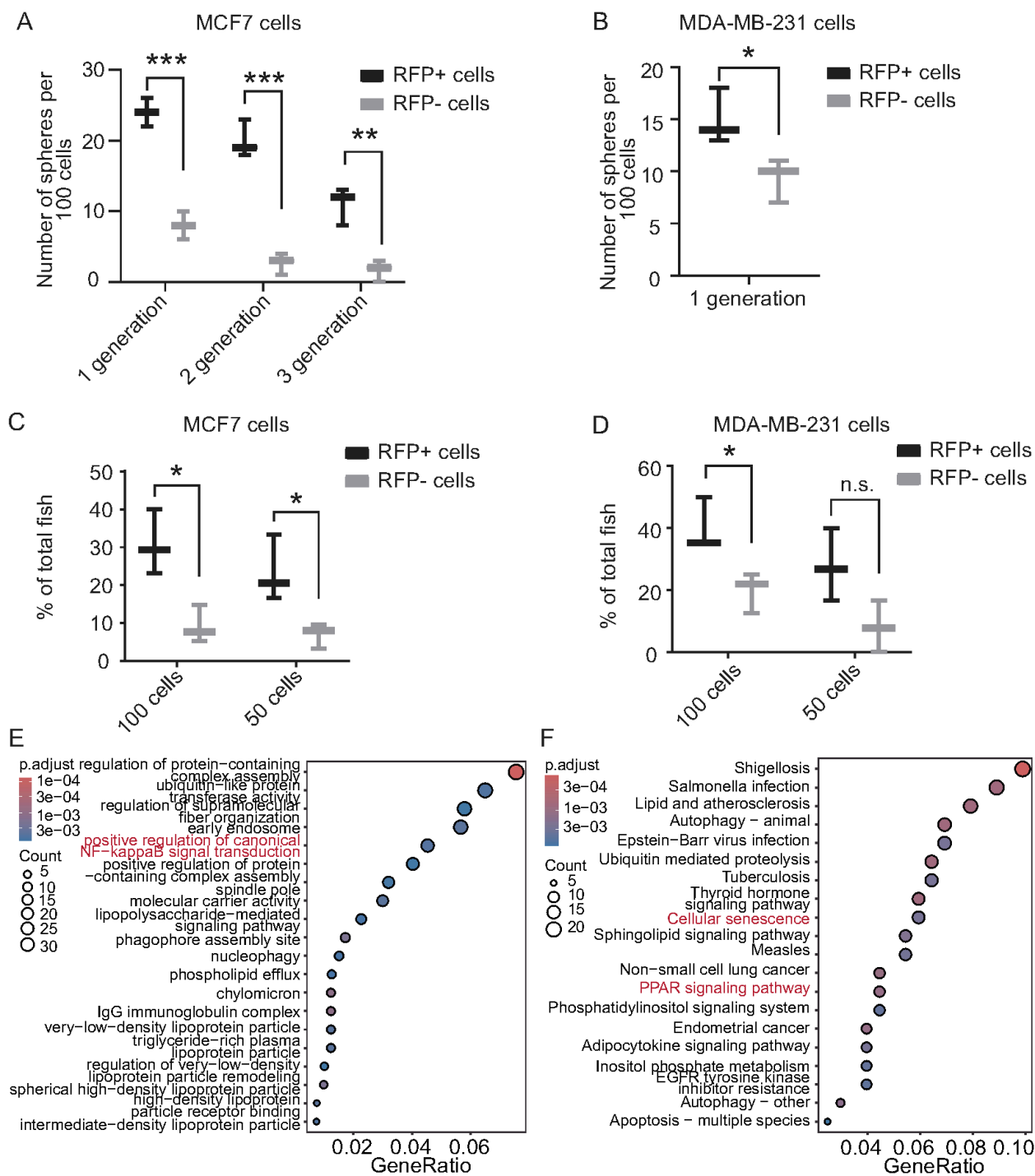


Figure 2

RFP⁺ cells possess typical CSC properties, and the NF-κB signaling pathway is specifically activated in these CSCs.

(A) RFP⁺ and RFP⁻ MCF7 cells were sorted by FACS and cultured at a density of 100 cells per well under sphere-forming conditions. Sphere numbers were quantified after 7 days. For serial replating assays,

primary spheres were collected, dissociated into single cells, and replated at the same density of 100 cells per well. Data are shown as mean $\pm$ SEM with three independent replicates. **P < 0.01, ***P < 0.001.

(B) FACS-sorted RFP⁺ and RFP⁻ MDA-MB-231 cells were seeded at 100 cells per well for sphere formation. Sphere counts were determined 7 days after culture. Data are shown as mean $\pm$ SEM with three independent replicates. *P < 0.05.

(C, D) FACS-isolated RFP⁺ and RFP⁻ MCF7 (C) and MDA-MB-231 (D) cells were labeled with CM-Dil. A total of 50 or 100 labeled cells were transplanted into the yolk sac of Tg (kdrl:eGFP) zebrafish embryos. Tumor formation was assessed by confocal microscopy at 5 days post-transplantation. *P < 0.05.

(E, F) FACS-sorted RFP⁺ and RFP⁻ MCF7 cells were subjected to mass spectrometry to explore altered signaling pathways. Top 20 significantly enriched signaling pathways (E) and KEGG pathways (F) were identified based on differentially expressed proteins between RFP⁺ and RFP⁻ cell populations.

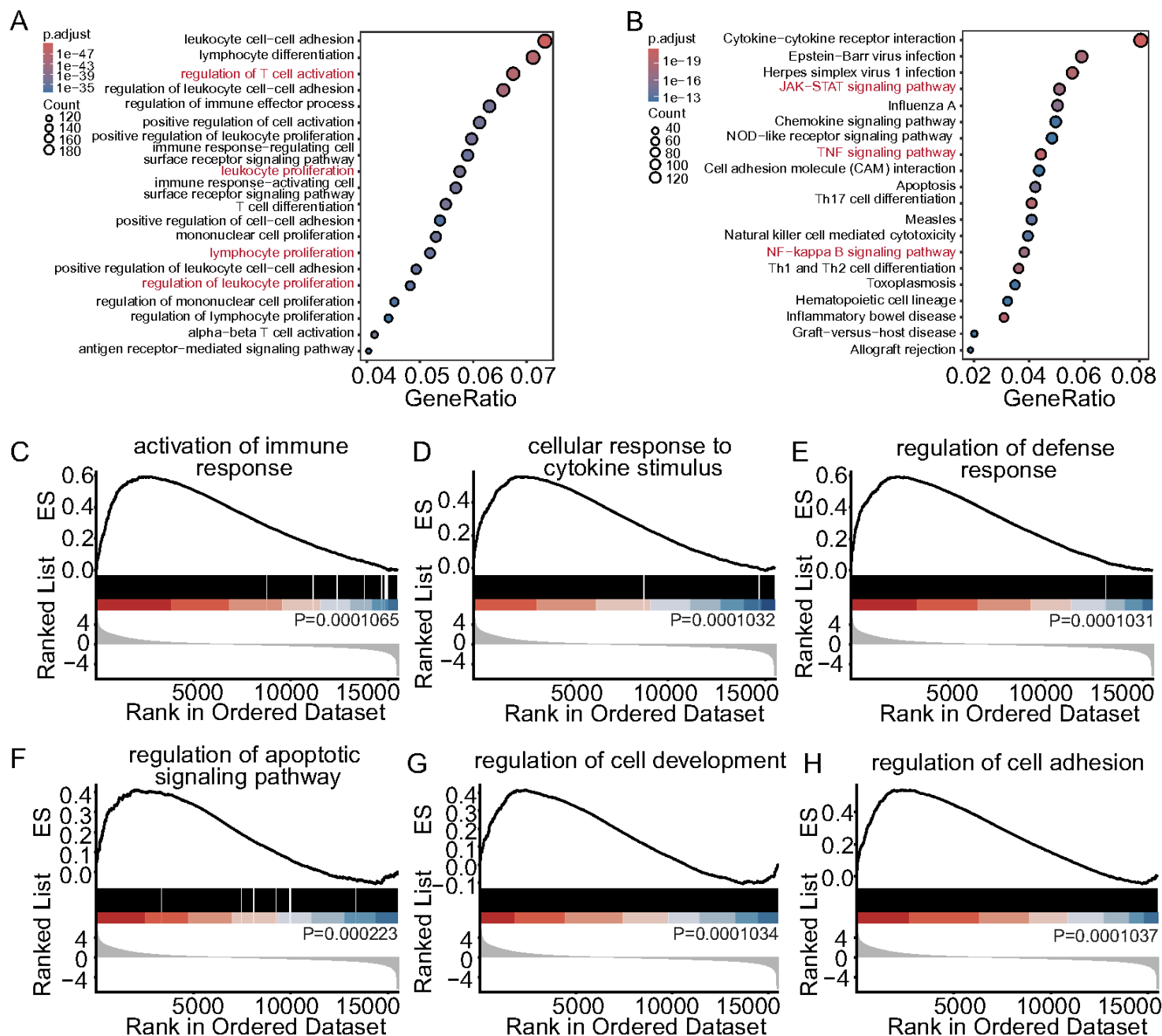


Figure 3

TROP2-targeted CAR-T treatment modulates immune-related biological processes in breast cancer cells *in vitro*.

(A) Top 20 enriched pathways based on enrichment analysis of differentially expressed genes between TROP2-targeted CAR-T and MOCK CAR-T treatment groups.

(B) Top 20 enriched KEGG pathways identified by KEGG enrichment analysis of differentially expressed genes between TROP2-targeted CAR-T and MOCK CAR-T treatment groups.

(C–H) Gene set enrichment analysis (GSEA) enrichment plots for the indicated gene sets: activation of immune response (C), cellular response to cytokine stimulus (D), regulation of defense response (E), regulation of apoptotic signaling pathway (F), regulation of cell development (G), and regulation of cell adhesion (H).

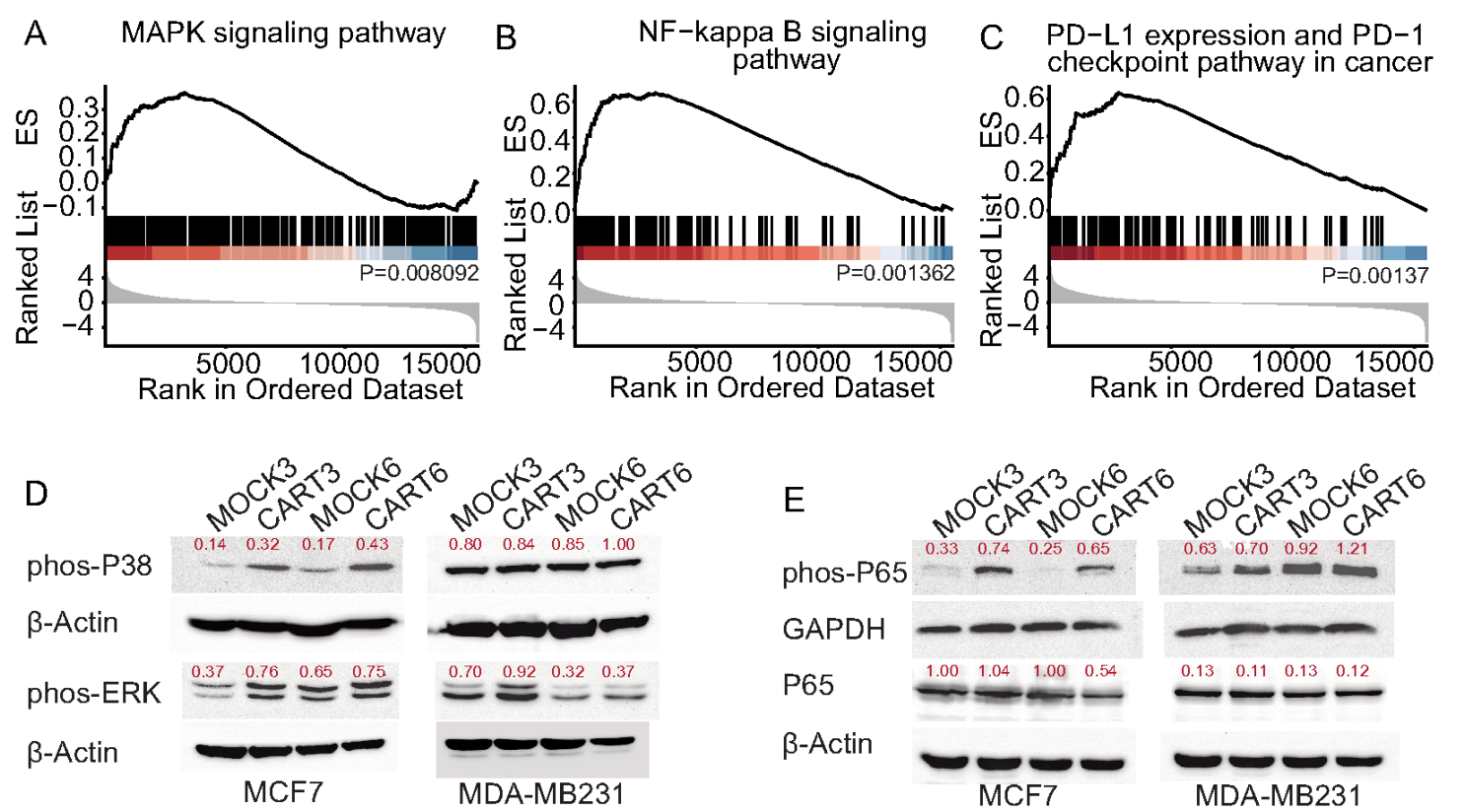


Figure 4

TROP2-targeted CAR-T treatment modulates the NF-κB signaling pathways in breast cancer cells *in vitro*.

(A–C) Gene set enrichment analysis (GSEA) enrichment plots for the MAPK signaling pathway (A), NF-κB signaling pathway (B), and the PD-L1 expression/PD-1 checkpoint pathway in cancer (C).

(D) Immunoblot analysis of phosphorylated P38 and ERK expression in MCF7 and MDA-MB-231 cells following TROP2-targeted CAR-T co-culture. Breast cancer cells were co-incubated with TROP2-targeted

CAR-T cells at an E:T ratio of 1:1 for MCF7 cells and 5:1 for MDA-MB-231 cells, respectively, for 3 and 6 days. β -Actin served as the loading control. E:T ratio, CAR-T cells to tumor cells.

(E) Immunoblot analysis of phosphorylated P65 expression in MCF7 and MDA-MB-231 cells after TROP2-targeted CAR-T intervention. Cells were co-cultured with TROP2-targeted CAR-T cells under the same E:T ratio and time-course conditions as in (A). β -Actin and GAPDH were used as loading controls. E:T ratio, CAR-T cells to tumor cells.

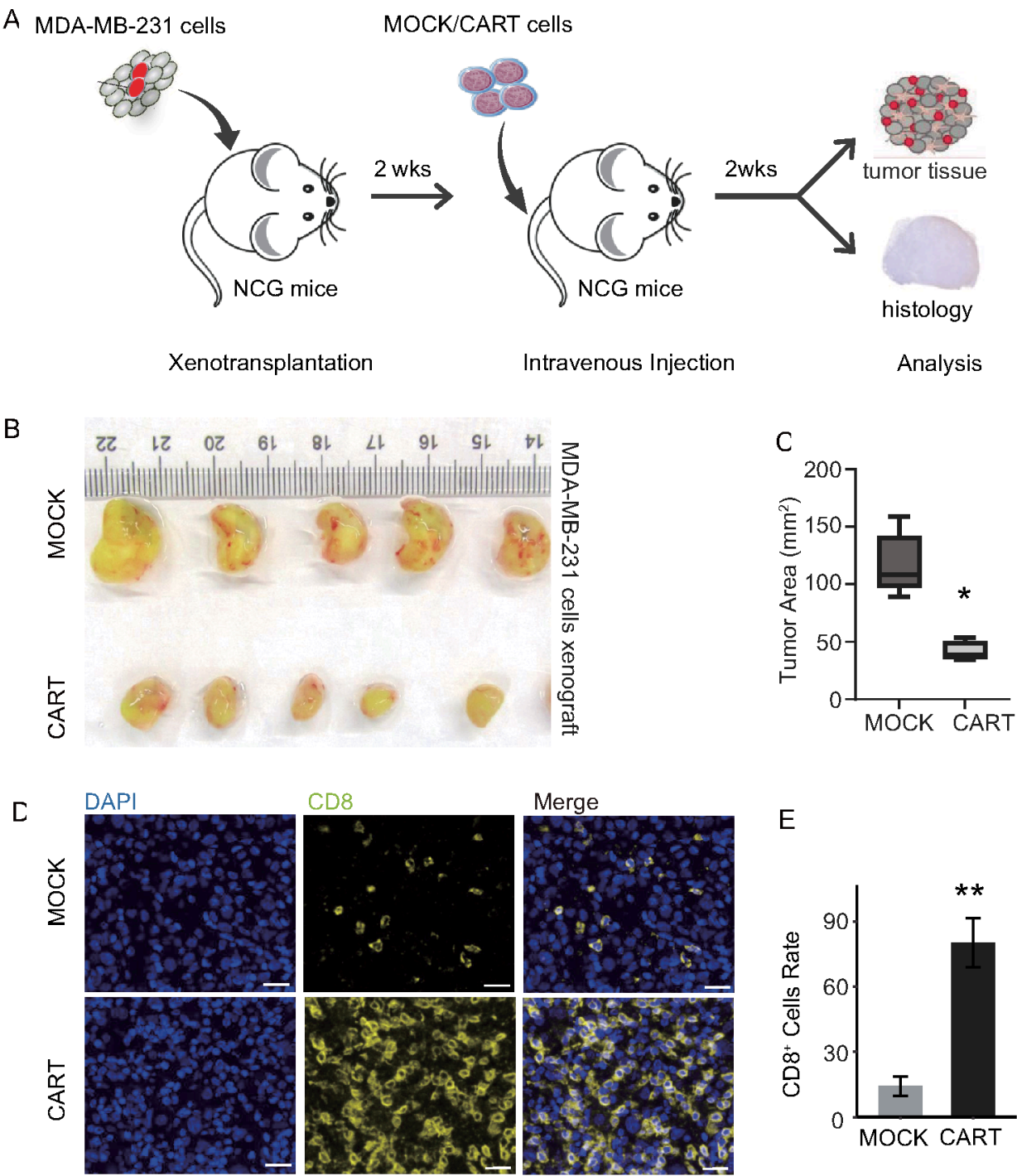


Figure 5

TROP2-targeted CAR-T cells efficiently suppress the growth of breast cancer xenograft tumors *in vivo*.

(A) Schematic overview of the *in vivo* experimental design. MDA-MB-231 cells were subcutaneously implanted into 6-week-old female NCG mice.

(B) Representative images of xenograft tumors from the two experimental groups (N = 5 mice per group).

(C) Quantification of tumor area in the two groups. Data are presented as mean $\pm$ SD. *P < 0.05.

(D) Immunofluorescence staining of tumor tissues using a CD8 antibody. Increased infiltration of CD8⁺ T cells was observed in the TROP2-CAR-T treatment group. Representative microscopic images are shown. Scale bar, 20 μ m.

(E) Quantitative analysis of CD8⁺ T cell abundance in the Mock and CAR-T groups. Data are presented as mean $\pm$ SEM. **P < 0.01.

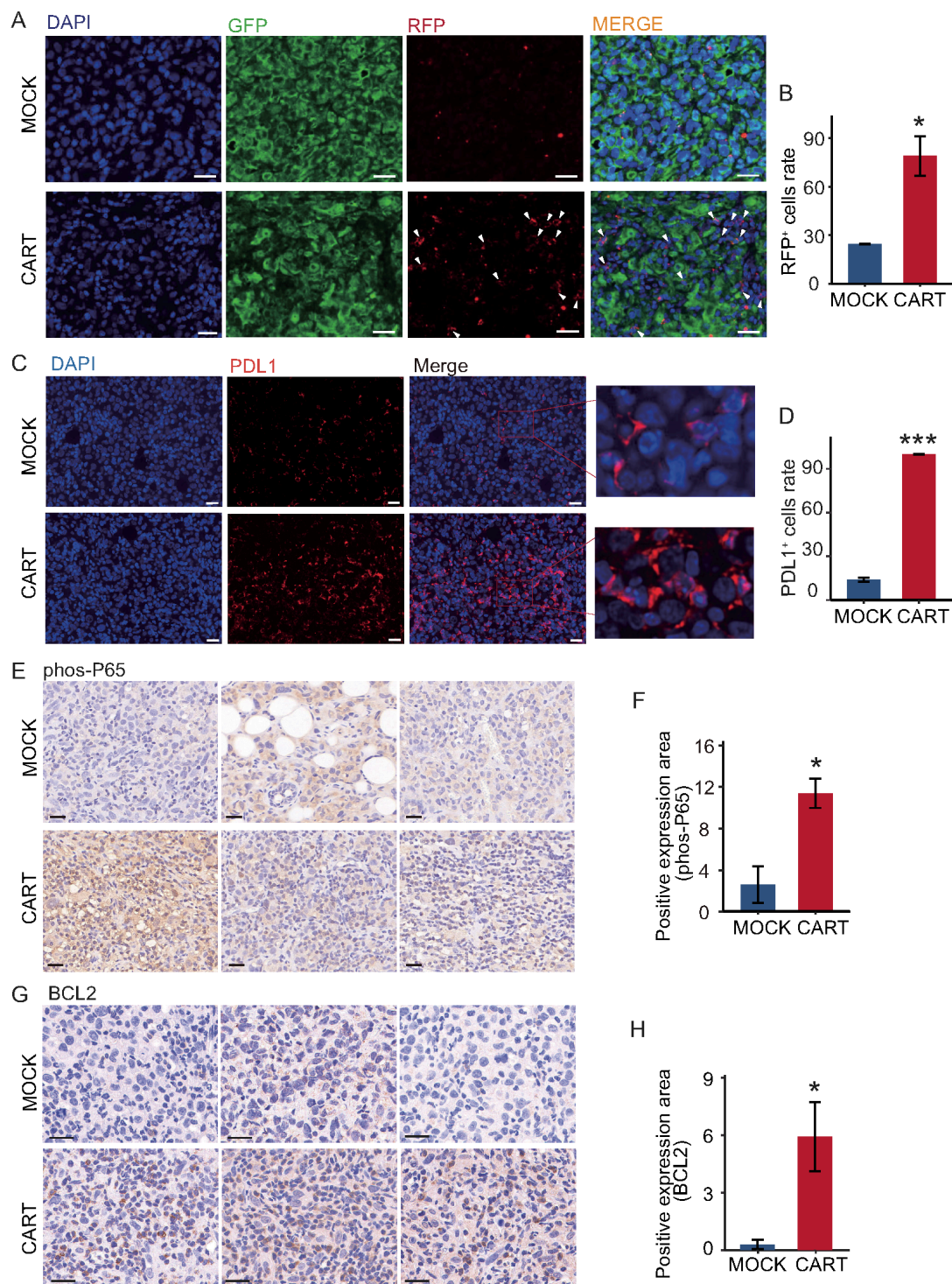


Figure 6

TROP2-targeted CAR-T treatment regulates the NF- κ B and apoptosis signaling pathways in breast cancer cells *in vivo*.

(A) Immunofluorescence staining of tumor tissues with an RFP antibody. Increased infiltration of RFP+ cells was observed in the TROP2-CAR-T group. Representative images are presented. Scale bar, 20 μ m.

(B) Quantitative analysis of RFP⁺ cell proportions in the Mock and TROP2-CAR-T groups. Data are presented as mean $\pm$ SEM. *P < 0.05.

(C) Immunofluorescence staining of tumor tissues with a PD-L1 antibody. Higher abundance of PD-L1⁺ cells was detected in the TROP2-CAR-T treatment group. Representative images are presented. Scale bar, 20 μ m.

(D) Quantitative analysis of PD-L1⁺ cell proportions in the Mock and TROP2-CAR-T groups. Data are presented as mean $\pm$ SEM. ***P < 0.001.

(E) Immunohistochemical staining of tumor xenograft using a phosphorylated P65 antibody to evaluate NF- κ B pathway activation in Mock and TROP2-CAR-T treatment groups. Representative images are shown. Scale bar, 10 μ m.

(F) Quantitative analysis of phosphorylated P65⁺ cells in the two groups. Data are presented as mean $\pm$ SEM. *P < 0.05.

(G) Immunofluorescence staining of tumor xenograft with a BCL2 antibody in Mock and TROP2-CAR-T treatment groups. Representative images are presented. Scale bar, 25 μ m.

(H) Quantitative analysis of BCL2⁺ cell proportions in the Mock and TROP2-CAR-T groups. Data are presented as mean $\pm$ SEM. *P < 0.05.