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BCL-2 mutant B7H6-CAR-T cells synergized with venetoclax for treating small cell lung cancer

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ABSTRACT

Background Patients with small cell lung cancer (SCLC) generally have a poor prognosis, with an exceptionally high proliferative rate and a strong propensity for early metastasis, indicating the urgent need for novel therapies. The development of chimeric antigen receptor (CAR) s targeting solid tumors is limited owing to the lack of target antigens and low efficacy. In this study, we aimed to discover new targets for SCLC CAR-T therapy and develop CAR-T-based combinational treatment against SCLC in preclinical models.

Methods The in vitro antitumor activity of B7H6-specific CAR-T cell was evaluated. Venetoclax-resistant B7H6 CAR-T cell were designed and the synergistic effect of venetoclax and B7-H6 CAR-T cells was tested in vitro and in vivo.

Result B7H6 is highly expressed in SCLC tumors. CAR-T cell against B7H6 displayed antigen-specific antitumor efficacy. BCL-2(D103E)-expressing CAR-T cells showed resistance to venetoclax-induced apoptosis. The combinational treatment of venetoclax and BCL-2(D103E)-expressing B7H6-targeting showed potent anti-SCLC effect in vitro and in vivo.

Conclusions Our findings suggest that the combination of BCL-2 mutant-expressing B7H6-targeting CAR-T cells and venetoclax could be a promising novel strategy against B7H6-expressing SCLCs and other solid tumors, providing the foundation for CAR-T cells and proapoptotic small molecules therapy in patients with SCLCs in a clinical trial.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Small cell lung cancer (SCLC) has a poor prognosis, and chimeric antigen receptor (CAR)-T therapies for solid tumors have been limited due to challenges like the lack of target antigens and low efficacy.

WHAT THIS STUDY ADDS

⇒ This study identifies B7H6 as a potential target for CAR-T therapy in SCLC and shows that BCL-2(D103E)-expressing CAR-T cells can resist venetoclax-induced apoptosis. The combination of venetoclax and BCL-2(D103E)-expressing B7H6-targeting CAR-T cells demonstrates synergistic antitumor effects in preclinical models.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE, OR POLICY

⇒ The study suggests a promising new strategy combining B7H6-targeting CAR-T cells with venetoclax, potentially offering a novel treatment for SCLC and other solid tumors in clinic.

BACKGROUND

Small cell lung cancer (SCLC), accounting for approximately 15% of lung cancer cases, is the most aggressive type of lung cancer, with a high degree of malignancy, rapid proliferation, rapid disease progression, and poor prognosis.[1 2] Radiotherapy, chemotherapy, immune checkpoint blockade, and combination therapies have been extensively used clinically for patients with SCLC.[3–8] SCLC has a median survival duration of less than 2 years for patients with early-stage disease and approximately 1 year for patients with metastatic disease and a 7% 5-year survival.[9 10] Therefore, new therapeutic methods should be urgently developed for SCLC.

Chimeric antigen receptor (CAR)-T cell therapies targeting CD19 and B-cell maturation antigen in hematological malignancies and multiple myeloma have achieved great clinical success,[11 12] but applications of CAR-T cells in solid tumors are restricted owing to tumor heterogeneity, lack of target antigens, inadequate infiltration, poor proliferation of CAR-T cells, and the immunosuppressive tumor microenvironment (TME). Researchers have exploited the efficacy of CAR-T targeting CD56 and delta-like ligand 3 (DLL3) to treat SCLC.[13 14] However, the antitumor activity of $CD56^+$ CAR-T cells is modest, and these can cause potential toxicity.[14] DLL3 CAR-T cells are currently tested in early clinical trials.[15] Exploring safe and effective tumor-specific antigens and designing novel CAR-T treatment strategies are critical for CAR-T therapies targeting SCLC.

B7H6, which binds to the natural killer (NK) cell-activating receptor NKp30, promotes the antitumor activity of NK cells.[16] While it is counterintuitive that high B7H6 expression in human glioma tissues and cervical cancer promotes tumor progression,[17 18] anti-B7H6 CAR-T cell therapy decreases the tumor burden in lymphoma, melanoma, and ovarian cancer models.[19 20] Notably, a clinical study revealed that 56.31% of SCLC tumor tissues were positive for B7H6 and high B7H6 expression was associated with shorter survival rates and poor prognosis in patients with SCLC,[21 22] indicating that B7H6 could serve as a promising therapeutic target for SCLC immunotherapy. However, the efficacy, safety profile, and potential resistance mechanisms of B7H6-targeting CAR-T therapy for SCLC have not yet been evaluated.

SCLC cells are suggested to express BCL-2 to avoid apoptosis, which provides a potential therapeutic target in these tumor cells. The recent inclusion of prosurvival BCL-2 family inhibitors in the arsenal of targeted therapies has demonstrated that the BCL-2 target is gradually being valued with its notable preclinical efficacy.[23 24] Venetoclax is a BH3 mimetic small-molecule drug that selectively targets BCL-2,[25 26] approved by the Food and Drug Administration (FDA) in 2018 as a single-agent therapy for relapsed or refractory chronic lymphocytic leukemia.[27] This finding highlights the potential for clinical evaluation of venetoclax in patients with SCLC with elevated BCL-2 expression.[28] However, the success of monotherapies has been limited due to the emergence of resistance mechanisms.

These challenges underscore the need for combination strategies to enhance therapeutic outcomes. Whether the promotion of tumor apoptosis by BCL-2 inhibitors synergizes with CAR-T therapy in SCLC remains unclear. Considering that exposure to BCL-2 inhibitors may lead to apoptosis of CAR-T cells during activation,[29] CAR-T cells with BCL-2 inhibitor-resistant molecules should be engineered to ensure the functional integrity of CAR-T cells during BCL-2 inhibitor treatment.[25 30 31]

In this study, we developed anti-B7H6 CARs to assess their antitumor effects alone and with venetoclax against SCLC in vitro and in xenograft models. Anti-B7H6 CARs showed specific activity against B7H6-expressing SCLC cells. To counter venetoclax's adverse effects, we engineered venetoclax-resistant CAR-T cells. Venetoclax synergistically enhanced CAR-T-mediated apoptosis in SCLC cells in vitro and in vivo, highlighting the potential of combining venetoclax-resistant CAR-T cells with venetoclax as a therapy for B7H6-expressing SCLC.

METHODS

Mice

NOD-Prkdc^{scid}IL2rγ^{tm1} mice were purchased from Shanghai Model Organisms Center. Female mice were chosen to minimize aggression and fighting. All mice were maintained under specific pathogen-free conditions. Animal care and use were in accordance with the institutional and National Institutes of Health (NIH) protocols and guidelines, and all animal-related experiments were approved by the Animal Care and Use Committee of Shanghai Jiao Tong University (Protocol: A2021005).

Cell lines and culture conditions

The Lenti-X 293 cell line was purchased from Clontech. SHP-77, NCI-H446, NCI-H82, NCI-H146, DMS53, DMS114, Raji, A549, CFPAC-1, K562, HepG2, Jurkat, and HeLa cells were provided by the Stem Cell Bank of the Chinese Academy of Sciences. Human skin fibroblast (HSF) was purchased from Shanghai Jingfeng Biotechnology. Human peripheral blood mononuclear cells (PBMCs) were purchased from AllCells Biotechnology Limited (Shanghai, China). 293, HepG2, HeLa, CFPAC-1, and A549 cells were cultured in complete Dulbecco's Modified Eagle's Medium (DMEM). Jurkat, K562, Raji, SHP-77, NCI-H446, NCI-H82, DMS53, DMS114, NCI-H146, and human primary T cells were cultured in complete Roswell Park Memorial Institute 1640 (RPMI 1640) (Gibco). DMEM and RPMI 1640 were supplemented with 10% Fetal Bovine Serum (FBS) (Gibco) and 1% penicillin/streptomycin (Thermo). All cells were cultured in a humidified incubator at 37°C with 5% CO_2.

Generation and production of lentiviral vectors

DNA fragments containing the single-chain fragment variable (scFv) recognizing human B7H6 (clone 47.39),[32] and NKp30 (aa 1–139), CD8 hinge, transmembrane domain 4-1BB, and intracellular domain CD3ζ were generated using overlapping PCR amplicons. Lentiviruses were produced by transient transfection of 293 cells with a four-plasmid system. Supernatants containing lentiviral particles were collected 48 and 72 hours post-transfection and concentrated by ultracentrifugation, as previously described.[33]

Transduction of human T cells

Total T cells were purified using an EasySep Human T Cell Isolation Kit (STEMCELL) from human PBMC. T cells were cultured in complete RPMI 1640 medium supplemented with 50 IU/mL interleukin (IL)-2 and 4 ng/mL IL-21. Anti-CD3 and CD28 were used to activate T cells. 2 days after activation, various CAR-containing lentiviruses were used to transduce T cells at a multiplicity of infection of 10, as previously described.[33]

Repetitive stimulation assays

Irradiated (100 Gy) Raji-B7H6 cells were used to stimulate CAR-T cells at a ratio of 3:1 (T: Raji-B7H6) once every 6 days during the entire culture period. On day 4 after stimulation, the CAR-T cells were used for cytotoxicity assays.

Flow cytometry

For cell surface staining, cells were resuspended in staining buffer (1×phosphate-buffered saline (PBS) with 1% FBS) with Fc-blocking reagents. Intracellular staining was performed following fixation for 30 min in

4% paraformaldehyde (PFA) and permeabilization with 1×Perm/Wash buffer (eBioscience) for 60 min. Cells were incubated with the indicated antibodies for 30 min. Cells were analyzed using the CytoFLEX Flow Cytometer (Beckman Coulter), and data were analyzed using the FlowJo software (Becton Dickinson). The antibodies used are listed in online supplemental table 1.

Western blot analysis

The cells were harvested and lysed using radioimmunoprecipitation assay lysis buffer (Cell Signaling Technology). Protein lysates were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. The antibodies used are listed in online supplemental table 1.

B7H6 analysis by quantitative PCR

Total RNA was extracted from cultured cells using the Total RNA Kit (Omega Bio-tek) and reverse transcribed into complementary DNA using the GoScript Reverse Transcription System (Promega). Quantitative PCR reactions were performed in triplicates using the SYBR Green Super Mix (Vazyme). The products were quantified using the 2-ΔΔCt method. Primers are listed in online supplemental table 1.

Transcriptomic RNA-sequencing

Approximately 1×10^6 cells DMS53 cells treated with the venetoclax (0, 20 nM, 100 nM) for 24 hours, 1×10^6 CAR-T cells treated with the venetoclax (0, 1 µM) for 4 days. After washing with cold PBS, the cells were lysed in 1 mL TRIzol solution for RNA isolation and sequencing. All data analysis and processing are conducted by Tsingke Biotechnology (Shanghai, China).

In vitro CAR-T cell cytotoxicity assay

CAR-T cells (1×10^5) were co-cultured with green fluorescent protein (GFP)-expressing tumor cells or tumor cells labeled with fluorescein isothiocyanate (FITC) fluorescent dye at various effector-to-target ratios (1:4, 1:2, 1:1, 1:0.5). 24 hours later, anti-CD3-PE and GFP or FITC were used to distinguish CAR-T cells and tumor cells, respectively, by flow cytometry. The specific killing was calculated by the formula: 100%-remaining tumor cells/untreated tumor cells. The cytokine interferon (IFN)-γ, tumor necrosis factor (TNF) and IL-2 produced in culture supernatants were quantified using CBA kits (BD Biosciences).

Animal models

Female NOD-Prkdc^{scid}IL2rγ^{tm1} mice were subcutaneously injected with SHP-77, NCI-H146, or DMS53 cells. After 7–10 days, the mice were randomly divided into different groups with CAR-T cells treated once intravenously. Vehicle or venetoclax (MedChemExpress) (25 mg/kg) was administered via oral gavage. For in vivo experiments, venetoclax was dissolved in 5% dimethyl sulfoxide (DMSO), 40% polyethylene glycol 300, 5% Tween-80, and 50% saline. Body weight and tumor dimensions were measured two times weekly, and tumor volume was calculated using the following formula: V=L×W×H/2, where L=length, W=width, and H=height of the tumor.

Statistical analysis

Data are expressed as the mean±SEM. Two-tailed unpaired Student's t-test was used to compare results for two groups. Comparisons among three or more groups were performed using one-way analysis of variance. P values were calculated using GraphPad Prism Software (V.8); $^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$ were considered statistically significant.

RESULTS

B7H6 is a potential CAR-T target for SCLC

We initially analyzed the expression levels of B7H6 in various types of tumor cells, including SCLC. We found that B7H6 showed high expression in K562, 293X, and NCI-H146 cells, moderate expression in SHP-77, NCI-H82, DMS53, and HepG2 cells, low expression in DMS114, HeLa, and Jurkat cells, and no expression in NCI-H446, A549, HSF, Raji, and CFPAC-1 cells (figure 1a and online supplemental figure S1a). Results were confirmed using western blotting and reverse transcription quantitative PCR (RT-PCR) (figure 1b,c and online supplemental figure S1b). Additionally, protein expression analysis from the Human Protein Atlas (https://www.proteinatlas.org/) (online supplemental figure S1c) and in silico analysis of transcriptional data from the The Cancer Genome Atlas (TCGA) and Cancer Cell Line Encyclopedia (CCLE) databases showed that B7H6 exhibits significantly higher expression in SCLC cell lines compared with normal tissues (online supplemental figure S1d and e). These findings indicate the potential of targeting SCLC with B7H6-specific CAR-T cells.

To generate a CAR specific to B7H6, we chose NKp30 and anti-B7H6 scFv as the targeting moiety (figure 1d). Both NKp30 CARs and anti-B7H6 CARs could efficiently transduce on human T cells (figure 1e). To determine whether the CAR construct affected T-cell subtype, we examined the T-cell lineage markers. NKp30 and anti-B7H6 CAR-T cells showed similar percentages of $CD4^+$ and $CD8^+$ T cells as nontransduced T (NT) cells (online supplemental figure S2a). At the end of CAR-T cells expansion, the proportions of PD-1^+, TIM-3^+, and LAG-3^+ cells were comparable (online supplemental figure S2b), the proportion of effector memory T cells (TEM, $CD62L^-CD45RO^+CD45RA^-$) was higher in anti-B7H6 CAR-T cells compared with NKp30 CAR-T cells, while central memory T cells ($CD62L^+CD45RO^+CD45RA^-$) were lower in anti-B7H6 CAR-T than NKp30 CAR-T (online supplemental figure S2c).

B7H6-specific CAR T cells mediate cytotoxicity against B7H6-expressing tumors

We examined the long-term proliferation of B7H6-targeting CAR-T cells from multiple donors and found that both CAR-T cells proliferated continuously (figure 2a,b).

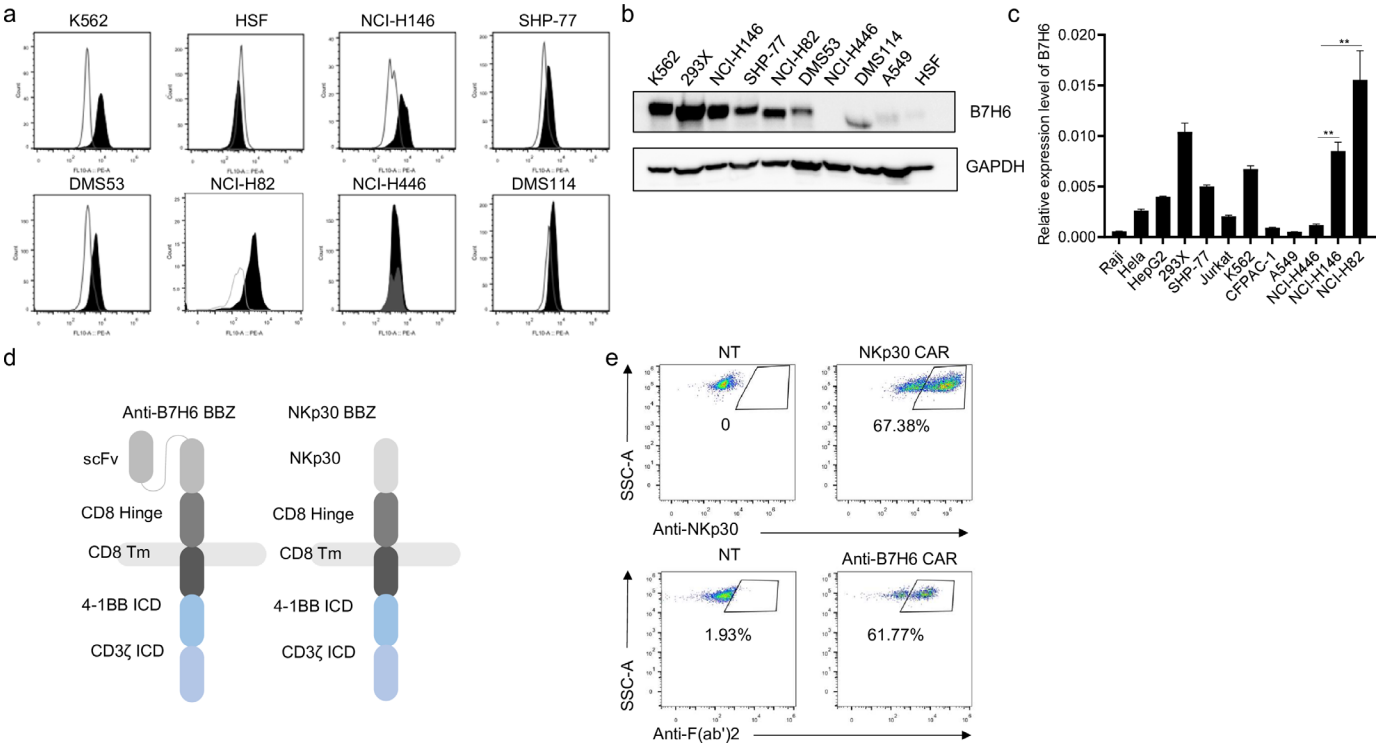

Figure 1 B7H6 is a potential CAR-T target for small cell lung cancer. (a) Flow cytometry analysis of B7H6 on different cancer cell lines and normal cells. The gray line, the isotype control; black shading, B7H6 staining. (b) Western blot analysis of B7H6 expression in different cancer cell lines and normal cells. Total lysate was separated using SDS-PAGE, followed by anti-B7H6 antibody staining. GAPDH was used as a loading control. (c) Analysis of B7H6 expression at the messenger RNA level in different cancer cell lines. GAPDH was used as a control. (d) Schematic diagram showing NKp30 CAR and anti-B7H6 CAR constructs. (e) Flow cytometric analysis of the proportion of cells positive for NKp30CAR (upper) or anti-B7H6 CAR (lower) in T cells expressing NKp30BBZ or anti-B7H6 BBZ. Data are presented as mean±SEM. Statistical analysis was performed using unpaired t-test, $^{**}p<0.01$. Representative results of three independent experiments. CAR, chimeric antigen receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ICD, intracellular cytoplasm domain; NT, nontransduced T; scFV, single-chain fragment variable; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

We observed that both CAR-T cells mediated robust cytotoxicity against B7H6$^+$ tumor cells compared with control NT (figure 2c, online supplemental figure S3a and S4a). B7H6-specific CAR-T cells mediated IFN-γ, TNF secretion (figure 2d, and online supplemental figure S3b and S4b), whereas IL-2 secretion was not significantly increased in CAR-T cells (online supplemental figure S4b and S5). CAR-T cells showed no difference in IFN-γ, TNF and IL-2 cytokine secretion compared with control NT when co-cultured with B7H6$^-$ normal cells. These results suggested that B7H6-targeting CAR-T cells efficiently lysed SCLC tumor cells in a B7H6 antigen-dependent manner.

B7H6-specific CAR-T cells suppress SCLC tumor growth in vivo

To study the antitumor activity of CAR-T cells targeting B7H6$^+$ cells in vivo, we established SHP-77 or NCI-H146 SCLC xenograft NSG mice models with NKp30 or anti-B7H6 CAR-T cells treated intravenously (figure 3a). NKp30 and anti-B7H6 CAR-T cells immunotherapy decreased the SHP-77 (figure 3b and c) and NCI-H146 (figure 3d,e and online supplemental figure S6a-h) tumor burdens. We also tested varying doses of anti-B7H6 CAR-T cells, and observed that different doses of anti-B7H6 CAR-T cells had similar antitumor activity (figure 3f and online supplemental figure S6i). These CAR-T cell therapies had no significant effect on the body weight of mice (online supplemental figure S7a–g), indicating a good safety profile. Despite initial success, the tumors relapsed following CAR-T therapy. A well-documented cause is antigen loss or reduction on cancer cells. As shown in figure 3g and h, the expression levels of B7H6 on NCI-H146 remained nearly unchanged before and after anti-B7H6 CAR-T therapy. This indicates that, in our in vivo models, relapse was not caused by antigen loss. These findings suggest that alternative mechanisms underlie tumor relapse and CAR-T cell dysfunction after therapy. Further studies are necessary to elucidate these mechanisms and identify strategies to improve the durability of CAR-T cell therapy. We focused on anti-B7H6 scFv-based CAR-T cells for further experiments due to better antitumor effector than NKp30 CAR-T cells

Venetoclax combined with anti-B7H6 CAR-T cells inhibits SCLC tumor growth

Since resistance to apoptosis is one of the hallmarkers of cancer, we considered combination therapy with CAR-T cells and anti-apoptotic protein inhibitor

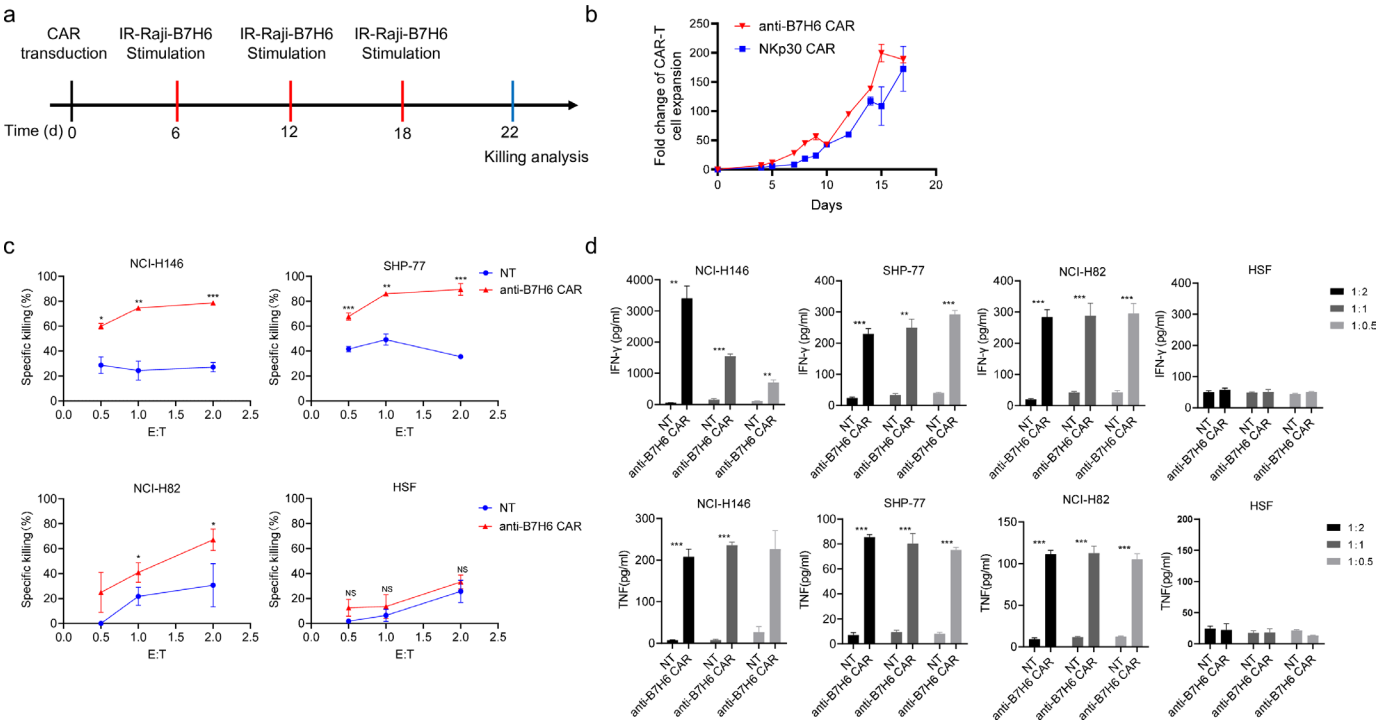

Figure 2 B7H6-specific CAR T cells mediate cytotoxicity against B7H6-expressing tumors. (a) Schematic diagram of the long-term CAR-T cell expansion assay. T cells were infected with the indicated CAR lentiviruses and stimulated by co-culture with irradiated (IR) Raji-B7H6 cells once every 6 days. (b) Expansion of CAR cells after repeated stimulation with irradiated Raji-B7H6 cells. (c) Anti-B7H6 CAR-T cells were co-cultured with the indicated tumor cells in triplicates at different effector:target (T cell:tumor cell) ratios for 24 hours. $CD3^+$ $FITC^-$ (T) and $CD3^-FITC^+$(tumor) were analyzed by flow cytometry to determine cytotoxicity. (d.) Cytokines were examined using Cytometric Bead Array (CBA) assays. Statistical significance was determined by unpaired t-tests. Data are presented as mean±SEM. $^{**}p<0.01$, $^{***}p<0.001$. Representative results of three independent experiments. CAR, chimeric antigen receptor; E:T, effector-to-target; FITC, fluorescein isothiocyanate; NT, nontransduced T.

venetoclax. We first investigated whether BCL-2, the direct target of venetoclax, was expressed in SCLC cell lines. We found that DMS53 cell expressed high levels of BCL-2, SHP-77 and NCL-H146 cells expressed medium levels of BCL-2, NCL-H446 and NCL-H82 expressed low levels of BCL-2, DMS114 cell line did not express BCL-2 (figure 4a and online supplemental figure S8a and b), consistent with data from the Human Protein Atlas (online supplemental file 1). We then tested the sensitivity of these cell lines to venetoclax. NCI-H146 and DMS53 were particularly sensitive to venetoclax (figure 4b). Venetoclax treatment led to a dose-dependent increase in active caspase-3 in NCI-H146 and DMS53 cells (figure 4c) and more apoptotic cells (Annexin V^+) in DMS53 cells (figure 4d). To investigate whether venetoclax treatment enhanced CAR-T cell-mediated tumor killing, we co-cultured anti-B7H6 CAR-T cells with NCI-H146 cells in the presence of venetoclax (figure 4e). Venetoclax combined with anti-B7H6 CAR-T cells led to a substantial increase in tumor killing compared with the vehicle group (figure 4f). However, our results indicated that CAR-T cell proliferation and IFN-γ secretion were significantly reduced in the presence of venetoclax (figure 4f). These findings suggest that venetoclax may impair CAR-T cell functionality, highlighting the necessity of further engineering venetoclax-resistant CAR-T cells to enable effective combination therapy with venetoclax.

Transcriptomic differences between control and venetoclax-treated tumor cells in vitro

To identify the potential genes or pathways involved in increasing CAR-T cells killing sensitivity, we profiled the whole transcriptome of venetoclax-treated tumor cells by RNA sequencing (RNA-seq) analysis. The volcano plots showed the 135 differentially expressed genes (DEGs) were detected by comparison of the venetoclax treatment and control groups (figure 5a). These genes were divided into subcluster 1, with 29 downregulated genes and subcluster 2, with 106 upregulated genes in the treatment group (figure 5b). The top 10 downregulated RNAs in venetoclax treatment groups were listed in (figure 5c). Cluster analysis revealed that the DEGs in subcluster 1 were mainly enriched in cell adhesion, signaling, activation, invasion, proliferation, survival, development and growth (figure 5c). We observed that genes related to the cell cycle, the cyclin G2, FLJ00132, MDA1a and septin-5, were decreased in venetoclax treatment groups (figure 5d). Biological process analysis revealed that the DEGs in subcluster 1 were mainly enriched in phosphatidylinositol 3-kinase signaling and positive regulation of

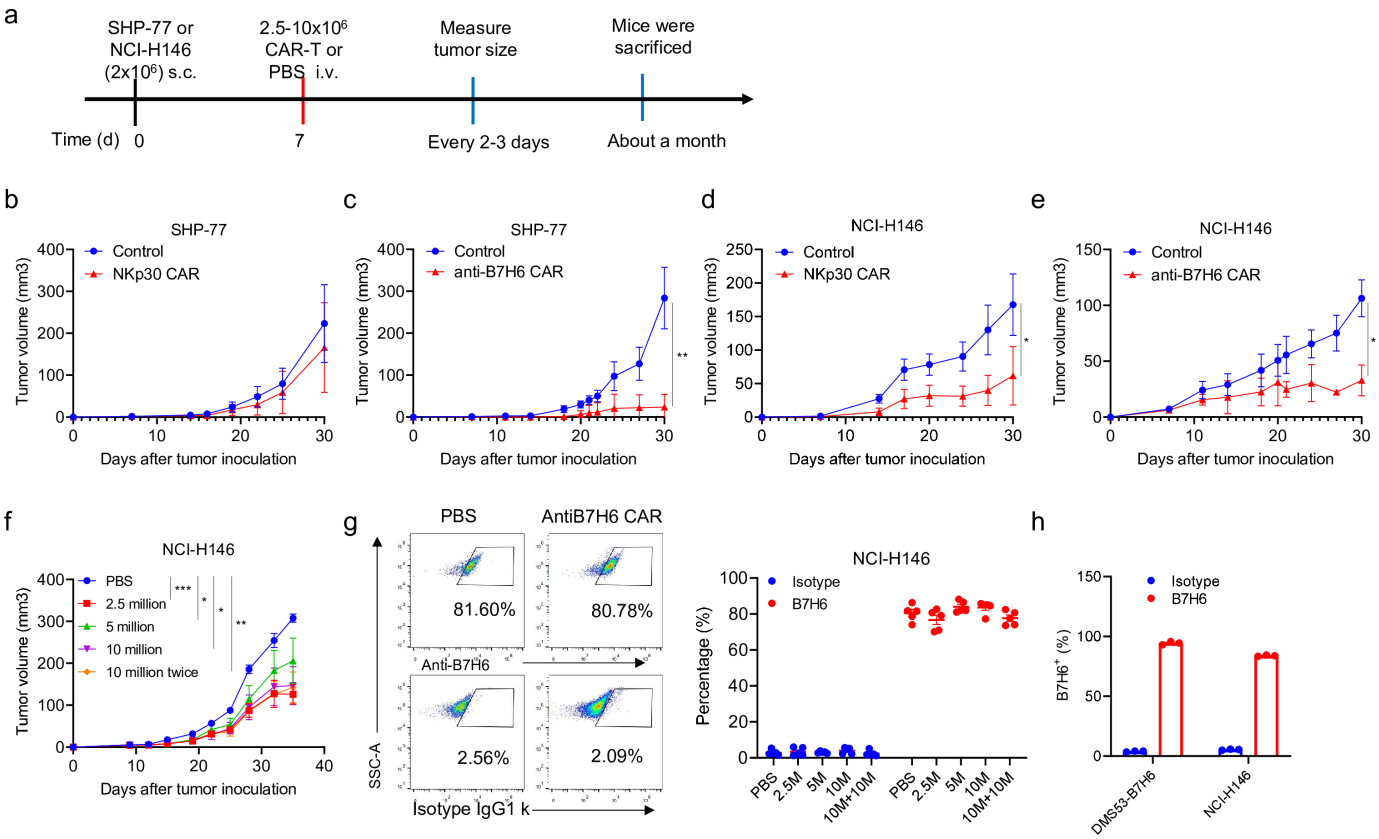

Figure 3 Targeting of B7H6 by CAR-T cells suppresses small cell lung cancer tumor growth in vivo. (a) Flowchart showing subcutaneous tumor xenograft models in NSG mice treated with NKp30 and anti-B7H6 CAR-T cells. (b–f) NSG mice (n=5–6) were subcutaneously injected with 2×10^6 SHP-77 (b and c); NCI-H146 cells (d, e and f). 7 days later, tumor-bearing mice were treated with 1×10^7 NKp30 CAR-T cells, 1×10^7 anti-B7H6 CAR-T cells or control untransduced T cells and tumor volume were recorded for NKp30 (b and d); anti-B7H6 CAR-T cells (c and e), varying indicated doses of anti-B7H6 CAR-T cells (2.5, 5, 10 million once and 10 million two times). (f) The expression of B7H6 on NCI-H146 cells from tumor-bearing mice was detected by flow cytometry on day 36 after tumor inoculation. (g and h) Expression of B7H6 on NCI-H146 before tumor inoculation. Data are presented as mean±SEM. Statistical analysis was performed using unpaired t-tests, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. Representative results of two independent experiments. CAR, chimeric antigen receptor; i.v., intravenous; PBS, phosphate-buffered saline; s.c., subcutaneous.

MAPK cascade (figure 5e), while those in subcluster 2 were mainly enriched in negative regulation of cell differentiation (figure 5f). Together, these findings highlight the signaling pathway alterations induced by venetoclax treatment, offering valuable insights into potential mechanisms underlying the enhanced sensitivity of SCLC to CAR-T cells when combined with venetoclax.

Modified anti-B7H6 CAR-T cells with resistance to venetoclax

Although the combination of anti-B7H6 CAR-T cells and venetoclax showed better tumor control than CAR-T cells alone, venetoclax caused a decrease in the number of anti-B7H6 CAR-T cells, and mediated a decrease in the IFN-γ secretion from anti-B7H6 CAR-T cells (figure 4f), indicating that venetoclax causes toxicity to CAR-T cells. We hypothesized that the overexpression of MCL-1, or mutant BCL-2 in CAR-T cells would enable them to resist venetoclax-mediated apoptosis. Thus, we engineered a lentiviral vector that encodes B7H6 CAR, MCL-1, and mutated BCL-2 (D103E and G101V) linked to a 2A peptide (figure 6a).[34] BCL-2 signaling enhances the expansion of the T-cell population following antigen recognition.[27 35] Consistently, BCL-2 and BCL-2 (D103E) CAR-T cells showed enhanced proliferation compared with that of anti-B7H6 CAR-T cells (figure 6b and online supplemental figure S9). Since the CAR expression of BCL-2, BCL-2(D103E), and BCL-2 (G101V)-CAR-T cells after stimulations was higher than MCL-1 CAR-T cells (80% vs 50%) (figure 6c), we focused on BCL-2 and mutant-expressing CAR-T cells in further experiments. The BCL-2 or mutant protein was significantly enriched in BCL-2, BCL-2 (D103E), and BCL-2 (G101V) CAR-T cells (figure 6d and online supplemental figure S10). We further tested whether anti-apoptotic proteins could affect the exhaustion and memory status of CAR-T cells. The proportion of CAR-T positive for programmed cell death 1 (PD-1), T cell immunoglobulin mucin (TIM-3), and lymphocyte activation gene 3 (LAG-3) was similar between the CAR-T cells examined (online supplemental figure S11a) and no significant differences were observed in the composition of effector memory T cells or central

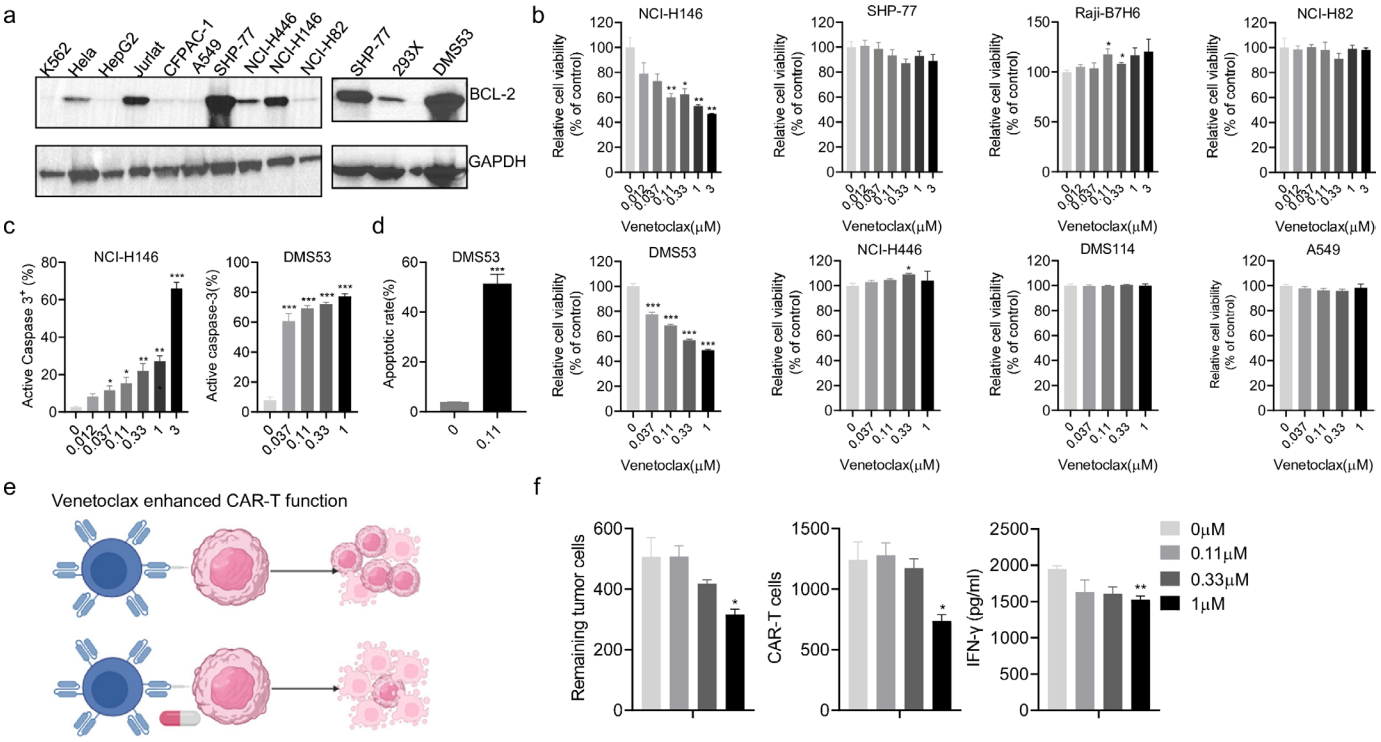

Figure 4 Venetoclax combined with anti-B7H6 CAR-T cells inhibits SCLC tumor growth. (a) Expression of BCL-2 in different cancer cell lines was determined by western blot. (b) Cell Counting Kit-8 (CCK8) assay of venetoclax-sensitive and venetoclax-resistant SCLC cell lines treated with venetoclax for 48 hours. (c) Apoptotic cells were stained for active caspase-3 by flow cytometric analysis of venetoclax-sensitive cells that were treated with various doses of venetoclax. (d) Apoptotic cells double-stained with Annexin V-FITC and Prodium Iodide (PI). (e) Proposal of CAR-T/venetoclax combination therapy to enhance CAR-T-mediated tumor killing. Quantification of NCI-H146 tumor killing by anti-B7H6 CAR-T cells in the presence of vehicle (DMSO) or different doses of venetoclax after 24 hours co-culture. Effector-to-target (E:T) ratios=1:2. (f) The tumor cells ($CD3^-FITC^+$) were analyzed by flow cytometry to determine cytotoxicity (left). Quantification of venetoclax-induced CAR-T cell toxicity on treatment with various doses of venetoclax in vitro by flow cytometry ($CD3^+FITC^-$) (middle). Cytokines secreted by anti-B7H6 CAR-T cells co-cultured with tumors and treated with various doses of venetoclax were measured by CBA assays (right). Data are presented as mean±SEM. Statistical analysis was performed using unpaired t-tests, $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. Representative results of two independent experiments. CAR, chimeric antigen receptor; CBA, Cytomic Bead Array; DMSO, dimethyl sulfoxide; FITC, fluorescein isothiocyanate; SCLC, small cell lung cancer.

memory T cells between unstimulated and repetitively stimulated cells (online supplemental figure S11b). Next, we evaluated whether CAR-T cells overexpressing anti-apoptotic proteins showed reduced apoptosis in the presence of venetoclax. We performed venetoclax CAR-T cell toxicity assays and observed that BCL-2(D103E)-overexpressing CAR-T cells were the most resistant to the venetoclax-induced suppression of cell proliferation (figure 6e and online supplemental figure S12). Thus, we focused on characterizing anti-B7H6 BCL-2(D103E) CAR-T cells in further experiments.

To assess whether overexpression of anti-apoptotic proteins impacts CAR-T cell killing capacity, we conducted extensive in vitro experiments against multiple SCLC cell lines. BCL-2(D103E) CAR-T cells exhibited enhanced cytotoxicity compared with anti-B7H6 CAR-T cells against $B7H6^+$ tumor cells (online supplemental figure S13a). Interestingly, despite the improved tumor-killing ability, BCL-2(D103E) CAR-T cells produced lower levels of cytotoxic effector molecules IFN-γ and TNF (online supplemental figure S13b). We next sought to investigate how overexpression of BCL-2(D103E) would affect CAR T-cell function in vivo. BCL-2(D103E) CAR-T cells showed slightly better antitumor effector than control CAR-T cells, but there was no statistical difference, these CAR-T cell therapies had no significant effect on the body weight of mice (online supplemental figure S14a and b). Also, B7H6 targeting CAR-T cells did not induce significant antigen loss of B7H6 in SHP-77 in vivo models (online supplemental figure S14c and d).

Since BCL-2(D103E)-overexpressed CAR-T cells performed better than control CAR-T cells in the presence of venetoclax, we investigated potential regulatory targets through the transcriptomic RNA-seq of these two types of CAR-T cells with or without venetoclax treatment. In the presence of venetoclax, 335 DEGs were detected by comparison of the control CAR-T cells and BCL-2(D103E) CAR-T cells (online supplemental figure S15a), with 113 downregulated genes with 222 upregulated genes in the BCL-2(D103E) CAR-T cells (online supplemental figure S15b). The top upregulated genes in BCL-2(D103E)

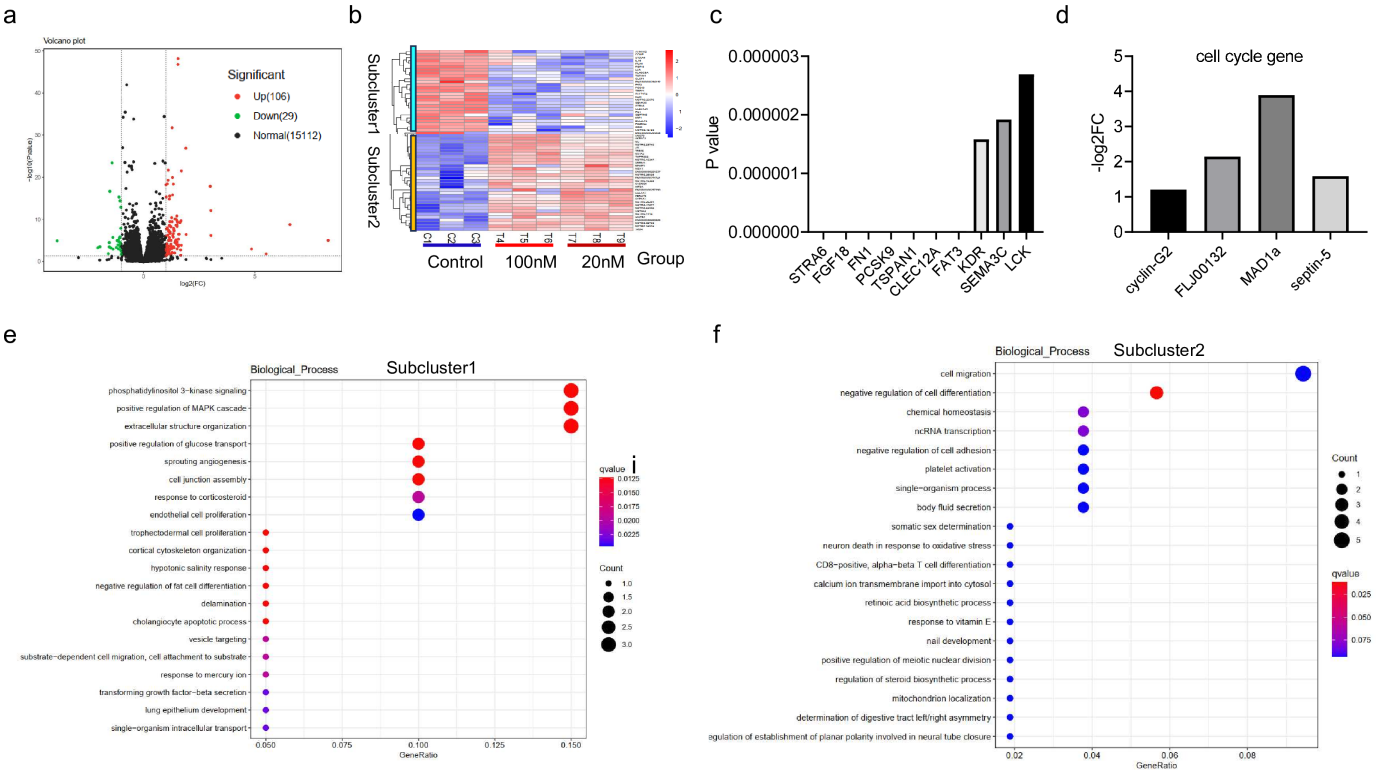

Figure 5 Transcriptomic analysis of venetoclax-treated tumor cells in vitro. (a) Volcano plots of differentially expressed genes (DEGs) in the venetoclax. (b) Subcluster analysis of the DEGs shown as a heatmap. (c) Top genes with significant downregulation. (d) The changed genes that downregulated in the venetoclax treatment were related to the cell cycle. (e–f) Biological process enrichment dotplot analyses of representative DEGs in subcluster 1, the control group (without venetoclax) (e), in subcluster 2, the venetoclax treatment group. (f) Representative results of two independent experiments.

CAR-T cells were listed in online supplemental figure S15c), including several genes that have been previously reported to be critically involved in cytokine receptors, cell proliferation and cell cycle regulation, such as CXCL13, CXCL11, IL-6, TNFSF13B, CXCL10, IFNB1, ISG15, IL1RN, AIM2, RIG1 and CDC14B. Biological process analysis revealed that the DEGs in BCL-2(D103E) CAR-T cells were mainly enriched in the cytokine-mediated signaling pathway (figure 6f). In the absence of venetoclax, BCL-2(D103E) CAR-T cells had similar clustering (online supplemental figure S16). These results indicated that BCL-2(D103E) CAR-T cells showed upregulation of pathways related to cytokine-mediated proliferation and apoptosis, which may explain the enhanced survival of CAR-T cells after venetoclax.

BCL-2(D103E)-overexpressing CAR-T cells synergized with venetoclax in vitro and in vivo

We explored the synergistic effect of BCL-2(D103E)-overexpressing CAR-T cells and venetoclax in killing the SCLC in vitro. Both CAR-T cells produced comparable IFN-γ and TNF (online supplemental figure S17). Venetoclax alone showed a dose-dependent DMS53 tumor cell inhibition ranging from 10% to 40% (figure 7a), BCL-2(D103E) CAR-T cells alone killed approximately 30% of the DMS53 tumor cells (figure 7a). Surprisingly, the combination of BCL-2(D103E) CAR-T cells and venetoclax showed a significant synergistic effect, leading to 80–90% tumor cell killing against DMS53. A similar synergistic effect was observed in NCI-H146 (figure 7a). Importantly, we observed that BCL-2(D103E) CAR-T cells not only maintained potent tumor-killing ability but also showed resistance to the proliferation arrest induced by venetoclax (figure 7b). We previously showed that venetoclax inhibited the production of IFN-γ by parental anti-B7H6 CAR-T cells (figure 4f). However, the levels of IFN-γ produced by BCL2(D103E) CAR-T cells were not affected by various dosages of venetoclax (figure 7c). To further test whether the expression level of B7H6 affects the synergistic effect between BCL2(D103E) CAR-T cells and venetoclax, we performed a similar cytotoxicity assay with SCLC cell lines with varying levels of B7H6 expression, with and without venetoclax. The data revealed a significant synergistic effect of BCL-2(D103E) CAR-T cells combined with venetoclax in killing SHP-77, DMS53, and NCI-H146 cells, BCL2(D103E) CAR-T cells were not affected by venetoclax (online supplemental figure S18a and b) and comparable IFN-γ and TNF secretion (online supplemental figure S18c). To investigate whether venetoclax could induce B7H6 expression and contribute to the synergistic effect, we measured B7H6 expression on multiple SCLC cell lines following 48 hours of venetoclax treatment. The results indicated that venetoclax did not alter B7H6 expression on B7H6^{+} tumor cell lines (online supplemental figure S19).

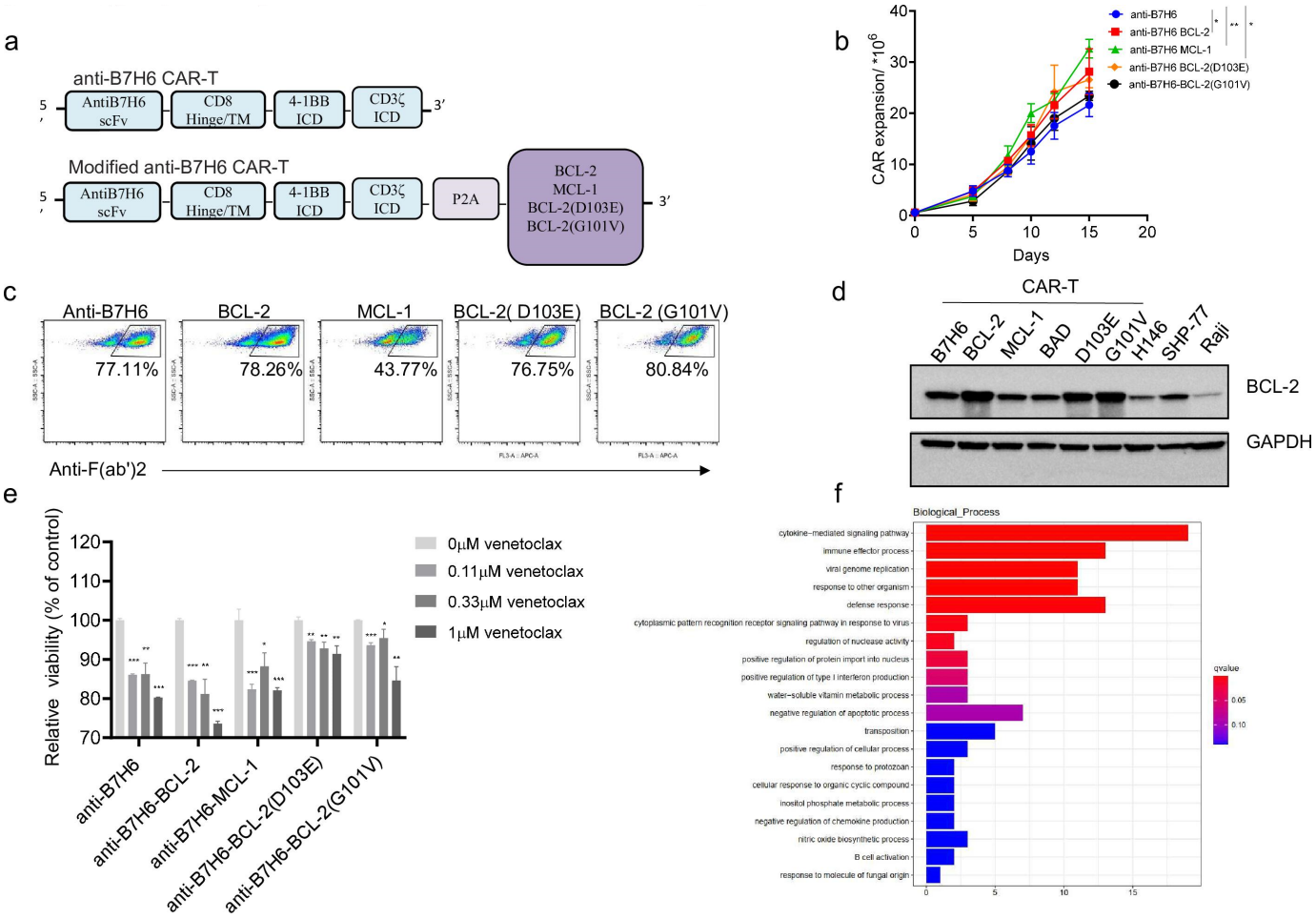

Figure 6 Modified anti-B7H6 CAR-T cells with resistance to venetoclax. (a) Construct designs for anti-B7H6 CARs resistant to venetoclax; the following proteins were overexpressed: wild-type BCL-2, MCL-1, BCL-2 with a D103E, G101V mutation. (b) The proliferation of indicated CAR-T cells was analyzed. (c) CAR expression of indicated anti-B7H6 CAR-T cells as measured by flow cytometry. (d) BCL-2 expression in different cells, as determined by western blotting; GAPDH was used as a loading control. (e) CCK8 assay results showing venetoclax-induced relative viabilities of CAR-T cells treated with various doses of venetoclax for 4 days in vitro. (f) Biological process enrichment dotplot analyses of upregulated differentially expressed genes in BCL-2(D103E) CAR-T cells treated with 1 μM venetoclax. Data are presented as mean±SEM. Statistical analysis was performed using unpaired t-tests, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. Representative results of three independent experiments (b–e), two independent experiments from two donors (f). CAR, chimeric antigen receptor; CCK8, Cell Counting Kit-8; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ICD, intracellular cytoplasm domain.

Finally, we tested the combined effects of BCL2(D103E) or anti-B7H6 CAR-T cells and venetoclax in vivo. NSG mice were engrafted with B7H6^{+} and venetoclax-sensitive SCLC tumor cells (DMS53). The mice were treated with BCL-2(D103E) CAR-T and anti-B7H6 CAR-T cells in the absence or presence of venetoclax (25 mg/kg; oral gavage). Both anti-B7H6 CAR-T and BCL-2(D103E) CAR-T cells alone showed moderate antitumor effects. Although venetoclax delayed tumor growth, the tumor relapsed later, and this finding is similar to the acquired resistance to venetoclax observed clinically. Notably, the combination of venetoclax and BCL-2(D103E) CAR-T cells significantly reduced tumor burden and delayed tumor relapse (figure 7d). The combination of venetoclax and control NT cells reduced tumor burden similarly to venetoclax alone (online supplemental figure 20b). CAR-T cell therapy resulted in a negligible loss of body weight, without notable toxicities (figure 7e and online supplemental figure 20b). At the end point of the experiment, the tumor-infiltrated BCL-2(D103E) CAR-T cells showed a significantly higher level of persistence than anti-B7H6 CAR-T cells in the presence of venetoclax (figure 7f), suggesting that BCL-2(D103E) CAR-T cells were resistant to venetoclax-induced apoptosis in vivo. To test whether the combinational strategy is effective against acquired resistance to venetoclax, we pretreated tumors with venetoclax until relapse. Indeed, the combination therapy resulted in better tumor control than venetoclax alone (online supplemental figure 20c). In summary, combination therapy using BCL2(D103E) CAR-T cells and venetoclax showed good antitumor efficacy against B7H6^{+} SCLC tumor cells.

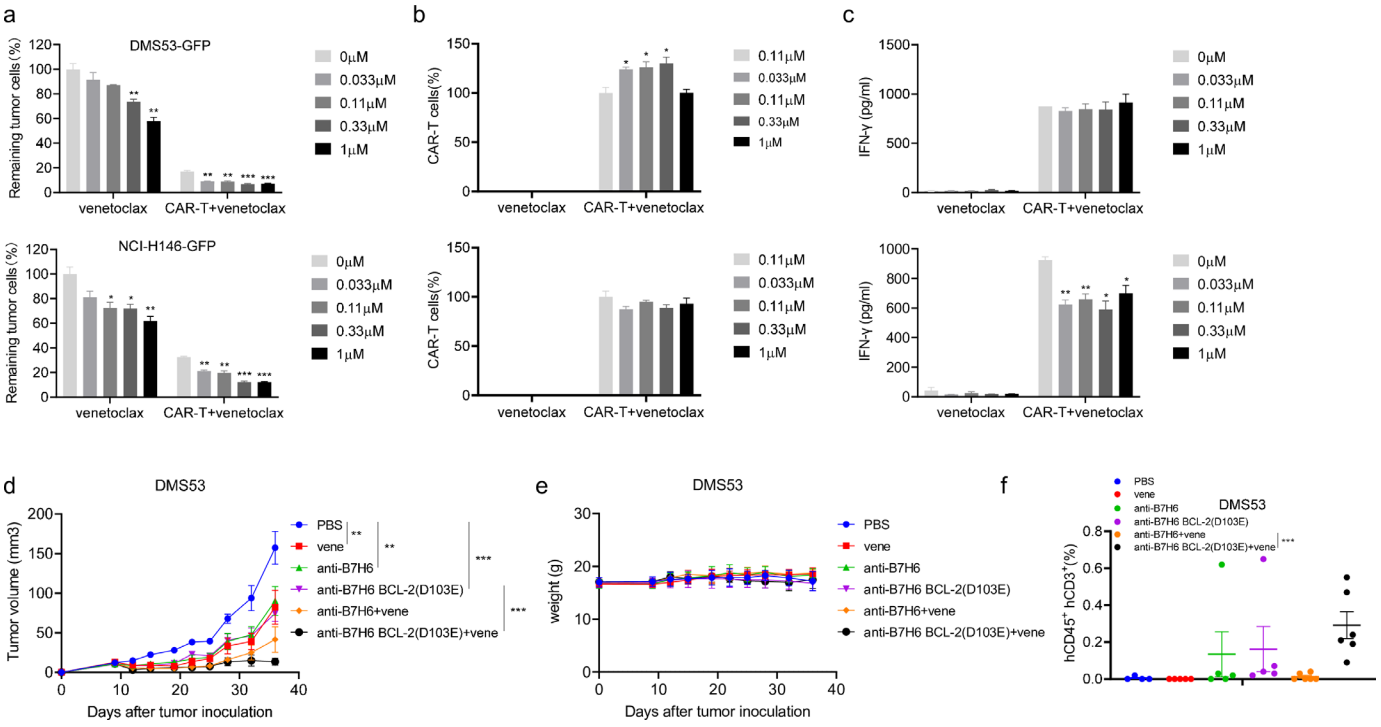

Figure 7 BCL-2(D103E)-overexpressed CAR-T cells synergized with venetoclax in vitro and in vivo. (a–c) DMS53-GFP (top) or NCI-H146-GFP (bottom) tumor cells were co-cultured with BCL-2(D103E) CAR-T cells for 24 hours in the presence of vehicle (DMSO) or various doses of venetoclax. Effector-to-target ratio=1:0.5. The tumor cells ($CD3^-FITC^+$) were analyzed by flow cytometry to determine cytotoxicity (a). Quantification of venetoclax-induced CAR-T cell toxicity after treatment with various doses of venetoclax in vitro by flow cytometry ($CD3^+FITC^-$) (b). Cytokines secreted by BCL-2(D103E) CAR-T cells co-cultured with tumors and treated with various doses of venetoclax were analyzed using CBA assays (c). (d–e) NSG mice were subcutaneously injected with 5×10^6 DMS53 cells. After 7 days, the mice were divided into six groups (n=5–6/group). Tumor-bearing mice were treated with vehicle, venetoclax, 1×10^7 anti-B7H6 CAR-T cells, 1×10^7 BCL-2(D103E) CAR-T cells plus either PBS or venetoclax. PBS and venetoclax (25 mg/kg) were administrated via oral gavage. Tumor growth curves (d) body weight (e) of all mice over were analyzed. (f) CAR-T cell ($mCD45^-hCD45^+hCD3^+$) persistence in the tumor was determined by flow cytometry after all the mice were sacrificed. Data are presented as mean±SEM. Statistical analysis was performed using unpaired t-tests, $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. Representative results of two independent experiments.CAR, chimeric antigen receptor; DMSO, dimethyl sulfoxide; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline.

DISCUSSION

Previous studies have revealed that B7H6 is not expressed in normal tissues but is expressed in various human tumor cell lines, including SCLC,[21] thus offering an attractive target for cancer immunotherapy. Our findings are consistent with those of a previous study, which showed that B7H6 is highly expressed in human SCLC tumor tissues and is correlated with long progression-free survival and increased total immune infiltrates ($CD45^+$) in patients.[21] Several CAR-T therapeutics targeting B7H6 have been developed. NKp30-CAR-T (NKp30-CD28-3ζ), demonstrated moderate antitumor effects. However, NKp30 receptors expressed by immature dendritic cells (iDCs) can lead to off-tumor activation of CAR-T cells, raising safety concerns.[32] To address this, anti-B7H6 scFv-based CARs were designed to avoid iDC-mediated activation while maintaining strong cytotoxicity against B7H6-positive tumor cells.[19 36] Further modifications introduced T-bet transcription factors to B7H6 CAR-T cells, enhancing their antitumor activity.[37] Despite these advancements, B7H6-targeted CAR-T therapy has yet to enter clinical trials, highlighting the need for further preclinical research. Here, we report that B7H6-targeting CAR-T cells demonstrate potent and specific tumor lysis activity without notable adverse effects, both in vitro and in vivo.

CAR-T cell therapy has demonstrated remarkable success in hematological malignancies but faces challenges in solid tumors, one of which is tumor heterogeneity.[10 38] Tumor-infiltrating CAR-T cells often upregulate inhibitory markers such as PD-1, TIM-3, and LAG-3, while tumor cells express high levels of immune checkpoints like programmed cell death ligand 1 (PD-L1).[39] Monoclonal antibodies targeting PD-1 and PD-L1 can restore CAR-T cell functionality, enhancing their ability to attack cancer cells. Combining CAR-T cells with immune checkpoint inhibitors has demonstrated improved efficacy and durability in preclinical models, with encouraging clinical trial results.[40–42]

The BCL-2 family proteins, such as MCL-1, BCL-2, and BCL-xL, are involved in anti-apoptosis regulation.[43] Studies have shown that high expression levels of BCL-2 are associated with poor prognosis of SCLC.[24] The recent addition of pro-survival BCL-2 family inhibitors

has revealed marked preclinical activity in SCLC. Unfortunately, in a phase II trial of a dual BCL-2 and BCL-xL inhibitor, navitoclax (ABT-263) showed a discouraging response, with a partial response of 2.6% and stable disease rate of 23%.[44 45] We hypothesized that inhibiting BCL-2 can not only directly suppress tumor cell growth but can also sensitize tumor cells to CAR-T cell-mediated cytotoxicity. As a proof of concept, we tested the efficacy and safety of the combination of venetoclax with CAR-T therapy in preclinical SCLC models. To avoid the pro-apoptotic effects of venetoclax on CAR-T cells, we engineered CAR-T cells to overexpress MCL-1, or mutant BCL-2, which have been reported to be associated with venetoclax resistance in tumor cells. We found that CAR-T cells overexpressing wild-type and mutated BCL-2 and MCL-1 proteins were less susceptible to apoptosis. Among these anti-apoptotic molecules, BCL-2(D103E) showed the greatest reduction in CAR-T cell apoptosis in vitro in the presence of venetoclax. Importantly, venetoclax and anti-B7H6 BCL-2(D103E) CAR-T cells showed a synergistic effect by killing SCLC cells in vitro. In xenografts, when combined with venetoclax, B7H6 CAR-T cells overexpressing BCL-2(D103E) showed higher and more durable antitumor activity than that observed with a single therapeutic agent, and the combination therapy significantly reduced tumor relapse compared with that observed with a single therapeutic agent. Moreover, other anti-apoptotic proteins (such as cFLIP, SIRT3, SIRT6, Bcl-xL or survivin) and pro-antiapoptotic proteins can be tested to reduce apoptosis in CAR-T cells. Testing whether anti-ferroptotic and anti-pyroptotic molecules contribute to CAR-T cell resistance to venetoclax would be interesting. Regarding a *BCL-2* mutation (Phe104Leu) that also reduces venetoclax binding to BCL-2,[25] we did not design a CAR cell that expresses mutant BCL-2 (Phe104Leu), and such a design in future experiments is also expected to reveal interesting results.

This study had some limitations. First, we did not test other inhibitors, such as the BCL-2, BCL-xL, and BCL-w inhibitors ABT-263 and ABT-737 or the MCL-1 inhibitor AZD5991. We focused on venetoclax because it is currently the only FDA-approved BH3 mimetic; the combination of other inhibitors with CAR-T cells will be considered in the future. Second, we only used established tumor cell line xenograft models, and primary SCLC xenograft models would be interesting to test in the future. In our study, we used subcutaneous tumor models, which are widely favored due to their ease of establishment, rapid growth, and reliability, offering valuable data for prioritizing drug candidates. However, subcutaneous models lack the complex TME of the original tissue.[46] Orthotopic models better mimic clinical disease and improve the predictive value of preclinical findings. Similarly, metastatic models can specifically mimic the distant dissemination ability of tumor cells. To comprehensively evaluate the synergistic effects of CAR-T therapy and venetoclax, all these tumor models will be critical and are planned for future testing.

In conclusion, our results demonstrate that venetoclax not only had no adverse effects on the proliferation and cytotoxicity of anti-B7H6 BCL-2(D103E) CAR-T cells, but also enhanced the sensitivity of SCLC cells to B7H6 CAR-T cells. The synergistic effect of venetoclax and anti-B7H6 BCL-2(D103E) CAR-T cells significantly reduced tumor burden and delayed tumor relapse in SCLC xenograft models. Our study showed that synergization of B7H6-targeting CAR-T cells with venetoclax could serve as a potential treatment for patients with B7H6-positive SCLC.

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