SUPPLEMENTARY MATERIALS AND METHODS

Animal study

C57BL/6J mice were obtained from Vitalstar Biotechnology (Beijing, China). To elucidate the molecular basis underlying the early-stage of HCC, we employed HTVi of pT2/shP53/luciferase and pT/Caggs-NRasV12 combined with pPGK-SB13 to establish a murine model of HCC in C57BL/6J mice. Additionally, a subcutaneous tumor model was established to characterize the growth of murine HCC Hepa1-6 cells. The shP53/NRas/SB model was produced using 15 μg pPGK-SB13 plasmid, 30 μg pT/Caggs-NRasV12 plasmid, and 15 μg pT2/shP53/luciferase plasmid. Plasmids of pPGK-SB13, Caggs-NRasV12 and pT2/shP53 were designed and synthesized by Tsingke Biotech Co. We monitored tumor development through *in vivo* luciferase imaging and weekly dissection in the pre-experiments. Mice were fixed in a fixation frame at 6–8 weeks of age. The shP53/NRas/SB plasmids were combined with 2 mL saline and administered to each mouse within 5 seconds. From 6 weeks onwards, animals received weekly injections of D-luciferin potassium salt (150 mg/kg HY-12591A MCE) and were placed in a small animal live imaging system to monitor tumor growth. From 6 weeks onwards, mice were also dissected weekly for observation of liver tumor growth. During the pre-experiment, it was noted that at week 8, the liver showed small white tumor foci (maximum tumor diameter ≤2 mm). At week 9, the maximum tumors grew to ≤5 mm. At week 10, tumors continued to grow but remained smaller than 10 mm in diameter. However, from 11–13 weeks, numerous large nodules emerged on the liver (maximum tumor diameter ≥10 mm), while mice developed ascites and showed cachexia. Therefore, we designating the 8–9 weeks period as early-stage HCC and the 11–13 weeks as late-stage HCC, characterized by significant increases in tumor size and the emergence of larger nodules on the liver. Spleens and tumor tissues of male C57BL/6J mice were harvested at 8, 9, 11, and 13 weeks for subsequent analysis.

CD36$^{-/-}$ mice were generated by Cyagen Biosciences. In the subcutaneous tumor model, male WT C57BL/6J mice were subcutaneously injected with 5×10^6 Hepa1-6 cells, and spleen and tumor tissues were collected 1 and 2 weeks post-injection. Two weeks following subcutaneous loading, tumor tissues from CD36$^{-/-}$ mice were collected for flow cytometric analysis, while blood was drawn for serum lipid oxidation assays.

All mice were fed a standard AIN-76A diet containing 50 mg/kg of iron (Research Diets, Inc.) unless stated otherwise. To explore the *in vivo* effects of iron overload, we fed mice with high-iron diet (500 mg/kg ferric citrate, TP0452G, Trophic Animal Feed High-Tech Co., Ltd, China). The high-iron-diet mouse models were established as follows: WT C57BL/6J male mice were separated into two groups: one group was fed a regular diet, while the other group was fed a high-iron diet (ferric citrate 500 ppm, Trophic Animal Feed High-tech Co., Ltd.) for 16 weeks. Following the dietary regimen, 5×10^6 Hepa1-6 cells were injected subcutaneously. After 7 days, mice were euthanized, and tumor and spleen tissues were collected for flow cytometric analysis (BD Biosciences).

CD8a-cre mice were generated by Cyagen Biosciences. To elucidate the contribution of caNRF2 in rescuing CD8$^+$ T cells infiltrating early-stage HCC, we engineered an adeno-associated virus (AAV) designated rAAV-CMV-DIO-flag-caNRF2-WPRE-hGH polyA, alongside a control virus, rAAV-CMV-DIO-flag-GAPDH-WPRE-hGH polyA. These viruses were designed to facilitate caNRF2 overexpression upon Cre-mediated activation. Mice were subcutaneously injected with 5×10^6 Hepa1-6 cells. Tumor volume was measured at 2-day intervals, starting from day 5. Once tumor margins became visible and slightly elevated, 2 μl of either rAAV-CMV-DIO-Flag-caNRF2-WPRE-hGH pA or rAAV-CMV-DIO-Flag-GAPDH-WPRE-hGH pA virus (2×10^{12} vg/mL, BrainVTA Co., Ltd.) was injected intratumorally. After 14 days, tumor, spleen tissues, and serum were collected for subsequent analysis.

C57BL/6J mice were subcutaneously injected with 5×10^6 Hepa1-6 cells. Tumor size was measured at 2-day intervals from day 4, with size calculated using the formula: tumor size = length × (width^2) /2. Concurrently, CD8$^+$ T cells were isolated from OT-1 mouse spleens and activated using CD3/CD28 antibodies (Ultra-LEAF™ Purified anti-mouse CD28 102116 Biolegend, Ultra-LEAF™ Purified anti-mouse CD3ε 100340 Biolegend) with 2 μg/mL OVA peptide under IL-2 (200-02-10UG PeproTech 50 IU/mL) cell-polarizing conditions for 4 days. OT-1 CD8$^+$ T cells were infected with caNRF2 lentiviral (Tsingke Biotech Co.) at a multiplicity of

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infection (MOI) of 20. Three days following infection, 5×10^6 $CD8^+$ T cells were injected into the tail vein of tumor-bearing mice. Blood samples were collected 5 days after transfer for subsequent analysis.

C57BL/6J mice were subcutaneously inoculated with 4×10^6 Hepa1-6 tumor cells at the flanks. Following a 7-day period to allow for tumor establishment, the mice were administered either IgG (Rat IgG2a isotype control; InVivo Selleck A2123) or anti-PD-1 antibody (Anti-mouse PD-1 (CD279); InVivo A2122) via intraperitoneal injection at a dose of 200 μg per mouse, with treatments being administered every other day. Throughout the experimental duration, tumor dimensions were meticulously monitored. On day 16, the mice were humanely euthanized, and blood samples were collected via orbital puncture, while tumors were excised for subsequent comprehensive analysis.

In mouse experiments, the level of significance was set at 5%, and the power was set at 80%. The least practicable difference between groups was set at no less than 25%. Generally, the expected coefficient of variation was 15%. The group size is about 6 animals/group for a 4-group experiment.

Cell line and cell culture

$CD8^+$ T cells were isolated from peripheral blood mononuclear cells (PBMCs) and the spleens of WT, $CD36^{-/-}$, and OT-1 mice. Cells were activated in RPMI 1640 medium containing 10% fetal bovine serum, 1% penicillin-streptomycin, and 2 mM glutamine. Activation was achieved using CD3/CD28 antibodies and IL-2 stimulator or 5 μg/mL OVA peptide (HY-P1489A MCE). Jurkat T cells and Hepa1-6 cells were obtained from ATCC and cultured in RPMI 1640 medium containing 10% bovine serum, 1% penicillin-streptomycin, and 2 mM glutamine. To elucidate the impact of iron accumulation on the dysfunction of $CD8^+$ T cells, we conducted experiments utilizing three distinct forms of iron and an iron chelator. Specifically, the iron compounds employed were ammonium ferric citrate (FAC; A9181, Solarbio), iron (III) chloride ($FeCl_3$; HY-Y0266, MCE), and iron (II) sulfate heptahydrate ($FeSO_4$; HY-Y1103A, MCE). Additionally, the iron chelator deferoxamine mesylate (DFO; HY-B0988, MCE) was utilized to investigate the effects of iron sequestration.

$CD8^+$ T cells isolation

Peripheral blood samples were collected from healthy donors. The samples were diluted 1:1 with serum-free medium and subjected to density gradient centrifugation using Ficoll solution to isolate PBMCs. Centrifugation was performed at 1,500 g for 20 minutes with acceleration and deceleration set to speed 1. The white blood cell layer was carefully aspirated, resuspended in serum-free medium, and subjected to further centrifugation for 8 minutes. Red blood cells were lysed, and cell counting was performed. Cells were either cryopreserved or used directly for $CD8^+$ T cells sorting and activated with CD3/CD28 antibodies under IL-2 cell-polarizing conditions.

Mouse splenic tissue was minced, homogenized into a cell suspension, and filtered. Red blood cells were lysed using a hemolysis solution, and the remaining cells were resuspended. CD8α antibody was added and incubated at room temperature for 30 minutes. After washing, $CD8^+$ T cells were sorted either on FACSAria™ III sorter or Fusion sorter (BD Biosciences).

Diaminobenzidine-enhanced prussian blue iron staining

Paraffin sections were deparaffinized and rehydrated. A wet chamber was preheated to 37°C for 30 minutes. The working solution was prepared according to the kit instructions (G1428-4 Solarbio) (steps A1 and A2, 1:1 ratio) and applied to cover the tissue, incubating at 37°C for 20 minutes. Sections were rinsed three times with distilled water and incubated in solution B at 37°C for 15 minutes. After washing, the working solution of C1, C2, and PBS (1:1:18 ratio) was added and incubated at 37°C for 20 minutes. Following further washes in PBS, sections were incubated in re-staining solution for 5 minutes and sealed with neutral resin. Imaging was performed using a Nikon DS-Ri2 microscope.

Serum iron levels measurement

Blood was collected and transferred to 1.5 mL centrifuge tubes, where it was left overnight at 4°C. Samples were centrifuged at 3,000 rpm for 10 minutes at room temperature to separate the serum. According to the manufactur-

er's instructions (BC1735 Solarbio), samples were treated with Reagent 1, Reagent 2, and chloroform. The mixture was shaken and centrifuged at 10,000 rpm for 10 minutes. Subsequently, 200 µl of the upper layer was transferred into a 96-well plate, and the absorbance was measured at 520 nm. Serum iron content (µmol/L) was calculated using the following formula: C standard solution × (A assay tube - A blank tube) / (A standard tube - A blank tube), where C is the concentration of the standard solution, and A is the absorbance of the assay tube.

Iron assay

Iron content was evaluated using an iron probe, FerroOrange (Fe^{2+} indicator MX4559 Maokang Bio). Cells were incubated with the FerroOrange for 30 minutes at 37°C, followed by flow cytometric detection (BD Fortessa). Fe^{2+} levels were calculated using PE MFI values. Data were analyzed using FlowJo 10 software.

Cystine deprivation in $CD8^+$ T cells

A customized RPMI medium deficient in cystine was prepared by Duoning Biological Company (Shanghai, China). Activated $CD8^+$ T cells were cultured in standard medium containing 200 µmol/L cystine or cystine-free medium (72 hours) and FAC (5 mM, 12 hours). After incubation period, the cells were harvested for subsequent flow cytometry analysis.

Serum malondialdehyde (MDA) assay

Blood was collected and transferred to 1.5 mL centrifuge tubes, where it was incubated at 4°C for 12 hours. Samples were then centrifuged at 3,000 rpm for 10 minutes at room temperature to obtain serum. According to the manufacturer's instructions (S0103M Beyotime), MDA working solution and standard dilutions were prepared. Each standard and serum sample (100 µl) was transferred into a separate 1.5 mL Eppendorf tube, followed by the addition of 200 µl MDA working solution. Samples were heated at 100°C for 15 minutes. After cooling, tubes were centrifuged at 1,000 g for 10 minutes, and 200 µl of the supernatant was transferred to a 96-well plate. MDA content was quantified at 532 nm using an ELISA reader (Epoch, Biotek) and calculated based on the standard curve.

Measurement of lipid peroxidation

Lipid peroxidation was determined using the Lipid Peroxidation Assay Kit (ab243377, Abcam). Cells were incubated with lipid peroxidation sensor for 30 minutes at 37°C, followed by flow cytometric detection (BD Fortessa), the degree of lipid peroxidation was assessed by the PE/FITC fluorescence ratio (decreased PE/FITC ratio indicates increased lipid peroxidation). Data were analyzed using FlowJo 10 software.

Detection of cell death

Cell death was assessed using Sytox Orange (S34861, Invitrogen). Cells were incubated with the Sytox probe for 30 minutes at 37°C, followed by flow cytometric detection. Data were analyzed using FlowJo 10 software.

Flow cytometry

Cell surface staining was performed at 37°C for 30 minutes. For intracellular staining, cells were stimulated with Cell Activation Cocktail (with Brefeldin A) (423303 Biolegend) for 6–12 hours, then fixed, permeabilized, and stained for intracellular molecules. All flow cytometry staining was conducted at 37°C for 30 minutes, followed by buffer washes prior to data acquisition. Flow cytometry was performed using a BD Fortessa flow cytometer. The FlowJo software (FlowJo, Ashland, Oregon, USA) was used for data analysis. After gating on the main population or $CD8^+$ T cell population in the raw data, further identification of positive and negative populations was performed based on the controls. Percentages or mean fluorescence intensity (MFI) were used for further analysis. All antibodies used for flow cytometry are listed below; antibodies included CD8α (PerCP/Cyanine5.5 anti-mouse CD8a 301032/PerCP anti-human CD8a 300906), TIM-3 (APC anti-human CD366 364804/APC anti-mouse CD366 134007), TIGIT (anti-human PE 372703/PE anti-mouse TIGIT 142104), PD-1 (anti-human FITC 329904/PE/Cyanine7 anti-mouse CD279 135216), IFNγ (anti-human APC 502512/FITC anti-mouse IFNγ 505806), TNFα (APC anti-human TNF-α 502912/APC anti-mouse TNFα), CTLA4 (APC anti-human 349908/APC

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anti-mouse CD152 106310), perforin (FITC anti-human Perforin 353310/PE anti-mouse Perforin 154306), and granzyme B (APC anti-human/mouse GranzymeB Recombinant 372204). These antibodies were purchased from Biolegend. These antibodies were purchased from Biolegend. The flow cytometry gating strategy is shown in Supplementary Figure 20.

Immunofluorescence (IF)

$CD8^+$ T cells were isolated from mouse spleens and treated with FAC (A9181 Sorlabio), oxLDL (YB-002 Yiyuan biotechnology), Ki696 (E1141 Selleck), or caNRF2 lentivirus. Cells were centrifuged at 1,000 rpm for 5 minutes using Tharmac Cellspin II apparatus to deposit them on slides. Cells were fixed with paraformaldehyde for 15 minutes, washed 3 times with PBS for 5 minutes each, permeabilized with 0.1% Triton X-100 for 10 minutes, and washed again three times with PBS. Blocking was performed using sheep serum for 30 minutes. Primary antibodies were incubated overnight at 4°C. Secondary antibody and DAPI staining were performed the following day. Imaging and analysis were conducted using a Leica Stellaris 5 confocal microscope. The following primary and secondary antibodies were used: anti-CEBPB (ab32358 Abcam dilution 1:500), anti-CEBPD (sc-515028 Santa Cruz dilution 1:50), anti-NRF2 (ab88957 Abcam dilution 1:150), anti-Alexa Fluor 488 donkey anti-mouse IgG (H+L) (A21202 Invitrogen dilution 1:200), and anti-Alexa Fluor 488 donkey anti-rabbit IgG (H+L) (A21206 Invitrogen dilution 1:200).

Immunohistochemistry (IHC) and hematoxylin-eosin (H&E) Staining

Tumor and adjacent normal tissues were excised, fixed in 4% neutral-buffered formalin, and embedded in paraffin for histological and immunohistochemical analysis. The following antibodies were used: anti-CD147 (ab188190 Abcam dilution 1:5000), anti-GPC3 (PA5-102528 Invitrogen dilution 1:100), anti-FAP (BMS168 Invitrogen dilution 1:200), anti-α-SMA (ab124964 Abcam dilution 1:1500), anti-CD31 (ab28364 Abcam dilution 1:50), and anti-CD3 (ab16669 Abcam dilution 1:250). Histological examination was performed using H&E staining to evaluate tissue samples from a pathological perspective. Images were captured using a Nikon DS-Ri2 microscope, and positive areas were analyzed using ImageJ software.

Multiplex IHC (mIHC)

Tissue samples from HCC patients were preserved in formalin and embedded in paraffin. All patients provided written informed consents to attend the study, which was approved by the Medical Ethics Committee of the First Affiliated Hospital of the Fourth Military Medical University (permission number: KY20172013-1). Formalin-fixed paraffin-embedded (FFPE) tissue sections, cut to a thickness of 4 μm, were baked overnight at 37°C, dehydrated in xylene, and rehydrated through a graded ethanol series. Antigen retrieval was performed in citrate buffer (pH=6) at medium-low temperature for 15 minutes. Sections were then washed three times in sterile water and TBST, followed by incubation with primary antibody for 1.5 hours at 37°C. Sections were then treated with a secondary antibody conjugated to streptavidin peroxidase for 10 minutes at room temperature to amplify the signal, followed by three washes in TBST. TSA dye was applied for 10 minutes at room temperature, followed by three washes in TBST. Remaining antibodies were stained using the aforementioned protocol, concluding with a final wash and sealing with a DAPI-containing mounting medium (P0301 Beyotime). Digital imaging was performed using an AKOYA Vectra Polaris system. The following primary antibodies were used: anti-CD4 (ab288724 Abcam dilution 1:100), anti-CD8 alpha (ab237723 Abcam dilution 1:100), anti-4 Hydroxynonenal (ab48506 Abcam dilution 1:100), anti-CD36 (ab252922 Abcam dilution 1:100), and anti-NRF2 (ab88957 Abcam dilution 1:100).

Electrotransformation

Jurkat T cells were harvested by centrifugation at 1,200 rpm for 3 minutes at room temperature. Cells were washed once with serum-free medium and centrifuged to remove the supernatant. Cells were then resuspended in 60 μl each of Electrotransformation Solution A and B. A total of 10 μg of siRNA (TSingke) was added to the suspension and mixed gently to avoid bubble formation. The mixture was transferred to an electrotransformation cuvette and subjected to an electric field of 530 V using an electrotrans-

fer apparatus (celetrix). Cells were promptly transferred into pre-warmed culture medium for further incubation. Specific sequences of the small interfering RNA (siRNA) used in this procedure were detailed in the accompanying documentation.

siMock: F-UUCUCCGAACGUGUCACGUTT
R-ACGUGACACGUUCGGAGAATT
siCEBPB-1: F-CCUCGCAGGUCAAGAGCAA
R-UUGCUCUUGACCUGCGAGG
siCEBPB-2: F-CUGCCUUUAAAUCCAUGGA
R-UCCAUGGAUUUAAAGGCAG
siCEBPB-3: F-CCCGUGGUGUUAUUUAAAGAA
R-UUCUUUAAAUAACACCACGGG
siCEBPD-1: F- CCGACCUCUUCAACAGCAA
R- UUGCUGUUGAAGAGGUCGG
siCEBPD-2: F- GCGCCUACAUCGACUCCAU
R- AUGGAGUCGAUGUAGGCGC
siCEBPD-3: F- CACAGACCGUGGUGAGCUU
R- AAGCUCACCACGGUCUGUG

Western blot analysis

Cells were washed with PBS and lysed using RIPA lysis buffer (P0013C Beyotime) supplemented with protease and phosphatase inhibitors according to the manufacturer's specifications. Lysates were centrifuged at 16,000 g for 30 minutes at 4°C to separate cellular debris. Supernatants were collected, and protein concentrations were determined using a Nanodrop 2000c (Thermo). Proteins were separated by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes after the addition of an appropriate volume of loading buffer. Membranes were blocked with 5% skimmed milk in TBST (HY-K1025 MCE) for 12–16 hours at 4°C with primary antibodies. Membranes were then incubated with secondary antibodies for 1 hour at room temperature. Protein bands were visualized using an enhanced chemiluminescent (ECL) method (P0018FS Beyotime) and a ChemiDoc Touch Imaging System (Bio-Rad). The following primary antibodies: anti-TFRC (ab214039 Abcam dilution 1:1000), anti-CD36 (SantaCruz sc-7309 dilution 1:500), anti-DMT1 (ab55735 Abcam dilution 1:1000), anti-ACSL4 (Huabio ET7111-43 dilution 1:1000), anti-p38 (phosphoT180+Y182) (ab195049 Abcam dilution 1:1000), anti-CEBPB (ab32358 Abcam dilution 1:1000), anti-CEBPD (ab245214 Abcam dilution 1:1000), anti-KEAP1 (ab227828 Abcam dilution 1:2000), anti-NFR2 (PA5-119633 Thermo dilution 1:500), anti-NQO1 (Invitrogen 39-3700 dilution 1:250), anti-HO-1 (ab13243 Abcam dilution 1:2000), anti-SLC7A11 (ab307601 Abcam dilution 1:1000), anti-GPX4 (ab12566 Abcam dilution 1:5000), anti-FSP1 (20886-1-AP proteintech dilution 1:4000), anti-IRP1 (9848R Bioss IRP1 dilution 1:1000), anti-IRP2 (4484R Bioss IRP2 dilution 1:1000), anti-Tubulin (66031-1-Ig proteintech dilution 1:10000), anti-Rabbit IgG (H+L) Secondary Antibody, HRP (31460 Invitrogen dilution 1:10000), anti-Mouse IgG (H+L) Secondary Antibody, HRP (31430 Thermo dilution 1:10000). The quantitative analysis was supplemented on top of each band.

Polymerase chain reaction

$CD8^+$ T cells were isolated from the spleens of WT and $CD36^{-/-}$ C57BL/6J mice and Jurkat T cells for polymerase chain reaction (PCR) analysis. Total RNA was extracted using the Total RNA Kit II, and reverse transcription was performed using the PrimeScript™ RT Reagent Kit (Perfect Real Time). The resulting cDNA was combined with TB Green® Premix Ex Taq™ II and amplified using a CFX-96 Touch Real-Time PCR Detection System (BIO-RAD). The mRNA expression of each gene was calculated using the $2^{-\Delta\Delta Ct}$ method. Primer sequences used for amplification were outlined in the relevant documentation.

CD36 (mouse): F-TGGGTTAAAACAGGCACCACT
R-CCAACAGACAGTGAAGGCTCA
TFRC (Human): F-GGCTACTTGGGCTATTGTAAAGG
R-CAGTTTCTCCGACAACTTTCTCT
GAPDH (mouse): F-TCTTCACCACCATGGAGAAG
R-GCAGAGATGATGACCCTTTTG
CEBPB (Human): F-CATCGACTTCAGCCCGTAC
R-GAGAAGAGGTCGGAGAGGAAG
CEBPD (Human): F-CCATGTACGACGACGAGAG
R-CTTGTGATTGCTGTTGAAGAGG
GAPDH (human): F-CTTCACCACCATGGAGAAG
R-GCAGAGATGATGACCCTTTT

Lentiviral overexpression of NRF2

$CD8^+$ T cells were transduced with an OE caNRF2 viral solution at a multiplicity of infection (MOI) of 20. Cells were harvested 4 days post-infection for subsequent assays.

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Lentiviral overexpression of CD36

$CD8^+$ T cells were isolated from the spleens of $CD36^{-/-}$ mice. Subsequently, these cells were transduced with a viral solution overexpressing CD36 at a multiplicity of infection (MOI) of 20. Four days post infection, the cells were harvested for further experimental assays.

Overexpression of CEBPB

293T cells were seeded in a 9 cm culture dish and incubated for 12-16 hours. Two 1.5-mL centrifuge tubes were prepared as follows. Tube 1: 250 μl Opti-MEM medium, 8 μg overexpression CEBPB plasmid, 6 μg packaging plasmid PsPAX2, and 2 μg packaging plasmid pMD2. G. Tube 2: 250 μl Opti-MEM medium and 10 μl Lipofectamine 2000 transfection reagent. The contents of tube 2 were added to tube 1, gently mixed, and incubated at room temperature for 20 minutes. The mixture was then added dropwise to the 293T cells. After a 6 hours incubation, the medium was replaced with fresh DMEM containing 10% fetal bovine serum. Following a 48 hours incubation, the viral supernatant was harvested and used to infect Jurkat T cells.

Co-culture of $CD8^+$ T cells with tumor tissue

A small piece of early-stage HCC tissue was excised and co-cultured with pre-activated mouse spleen $CD8^+$ T cells. After an 18 hours co-culture period, the expression of relevant molecules was analyzed using flow cytometry.

Transient transfection of OVA plasmid

When Hepa1-6 cells reached approximately 60% confluence in a 6-well plate, they were ready for transfection. Two 1.5-mL centrifuge tubes were prepared as follows. Tube 1: 200 μl Opti-MEM medium and 10 μl Lipofectamine 2000. Tube 2: 200 μl Opti-MEM medium and 2.5 μg OVA plasmid. The two tubes were left to stand for 5 minutes, then mixed together and incubated for another 20 minutes. After aspirating and discarding the original culture medium, 1.6 mL serum-free RPMI 1640 medium was added, followed by the transfection mixture. After 6 hours, the medium was replaced with 2 mL complete medium. After 48 hours, cells were subjected to G418 selection (Ant-gn-5 Invitrogen).

Luciferase assay

Mock Jurkat T cells and CEBPB-overexpressing Jurkat T cells were seeded in a 12-well plate for electroporation. Transfection of the 20 ng TFRC promoter (pGL3-TFRC) and 0.1 ng Renilla CMV constructs (pRL-CMV-Renilla) was performed. After a 24 hours incubation, cells were harvested into 1.5 mL Eppendorf tubes, and the supernatant was removed. Cells were lysed by adding 250 μl 1X Passive Lysis Buffer, followed by gentle shaking at room temperature for 15 minutes. Subsequently, 100 μl lysate was combined with Luciferase Reagent II to quantify firefly luciferase activity detected by a GloMax® 20/20 Luminometer (Promega, USA). Then, 100 μl stop reagent was added to measure Renilla luciferase activity. The ratio of firefly to Renilla luciferase activity was used to determine transfection efficiency (E1910 Promega).

ChIP assays and q-PCR

Chromatin immunoprecipitation (ChIP) was conducted in accordance with the manufacturer's protocol (Abcam ab500 ChIP Kit), utilizing the CEBPB polyclonal antibody (Proteintech 23431-1-AP) to selectively immunoprecipitate chromatin from Jurkat T cells. Subsequently, quantitative real-time PCR (qPCR) was employed to analyze 2% of each immunoprecipitated sample, thereby quantifying the amount of chromatin specifically bound by the antibody. The primers for the TFRC promoter sequence were shown as follows:

1: F-CACGAGCAGAATACAGCCAC
R-AGGTGCTTTCGGAGTCTTGA
2: F-AAAAGAGGGGAGGAGGCATC
R-ATTGTCTCTCCCAAGGACCG
3: F-AAAGTGCAGGGCTATGGAGT
R-AACGTGCGCCCTTTTCTATC
4: F-AACACTTCTCTTTGGGGAGAG
R-TGCAAGAAAGCAACCCATACC

ScRNA-seq sample collection

ScRNA-seq data from 4 patients pathologically diagnosed with HCC (patient ID: P10, P15, P16, P18, BCLC stage A, early stage) at Zhongshan Hospital Fudan University were reanalyzed in this study.[39] Single-cell RNA se-

quencing (scRNA-seq) was performed on three early-stage and three late-stage tumor samples from the HTVi HCC model. Briefly, single cells were encapsulated in droplets using the 10x Genomics GemCode Technology and processed according to the manufacturer's instructions. Libraries were generated and sequenced from the cDNAs with Chromium NextGEM Single Cell 3' HT Kit v3.1. Single cell encapsulation, complementary DNA (cDNA) library synthesis and RNA sequencing were completed by Gene Denovo (Guangzhou, China).

For bulk RNA-seq validation experiments, $CD8^+$ T cells were isolated from the spleens of wild-type (WT) C57 mice and subsequently activated in the presence or absence of ammonium ferric citrate (FAC; 5 mM) for 12 hours. Following activation, the cells were centrifuged at 400 rcf for 10 minutes at 37°C, washed once with phosphate-buffered saline (PBS), and resuspended in 1 mL of TRIzol reagent. The cell suspensions were homogenized by repeated pipetting to ensure complete lysis. The homogenized samples were immediately snap-frozen in liquid nitrogen and stored at –80°C until further processing. Briefly, the sample detection is carried out using the Illumina Novaseq X Plus.

ScRNA-seq data processing

The 10X Genomics Cell Ranger software (version 6.1.0) was employed to convert raw BCL files into FASTQ files. Subsequently, the alignment of FASTQ files to the Mus musculus reference genome (Ensembl release 106) was performed using the STAR algorithm, followed by gene count quantification. The cell by gene matrices for each sample were individually imported to Seurat[1] version 4.1.0 for downstream analysis.

As an additional quality control measure, cells meeting any of the following criteria were excluded: fewer than 300 or more than 5,400 unique genes expressed, fewer than 600 or more than 28,000 unique molecular identifiers (UMIs), and greater than 25% of reads mapped to mitochondrial genes. Doublet cells were identified and removed using DoubletFinder, which enhanced the identification of differentially expressed genes (DEGs). After filtering out unwanted cells, 2,000 highly variable genes were selected for downstream clustering analysis. Principal component analysis (PCA) was conducted, and cell types and subtypes were annotated based on the expression of known canonical marker genes characteristic of each cell type. Cell sub-clusters exhibiting similar gene expression patterns were annotated as the same cell type.

For cell type annotation, the log-normalized matrices were loaded into the SingleR R package, which annotates cell types by correlating the gene expression profiles of reference cell types with those of single cells. Initially, a Spearman correlation coefficient was calculated between the expression profile of each single cell and each reference cell type in the dataset. Subsequently, multiple correlation coefficients for each cell type were aggregated to generate a single value per cell type for each single cell. SingleR then reran the correlation analysis, focusing exclusively on the top cell types identified in the previous step. The analysis was restricted to variable genes to enhance accuracy. Ultimately, the cell type with the highest correlation value after the final run was assigned to the corresponding single cell. A total of 9 major clusters were identified among 41,359 cells obtained, Including T cells which positive for expression *Cd3d*, *Cd3e*, *Cd3g* (n=16,437), B cells identified by the expression of *Cd79a, Cd79b, Cd19* (n=4,025), plasma cells which expressed *Igkc, Jchain, Tnfrsf17* (n=1,248), macrophages which were positive for expression *C1qa, C1qc, clec4f* (n=6,939), neutrophils identified by the expression of *S100a8, S100a9, Csf3r* (n=8,432), HCC cells identified by the expression of *Alb, Apoa2, Afp* (n=1,990), endothelial cells defined by *Pecam1* and *Cd34* (n=1,429), mesenchymal cells marked by *Col1a1* and *Acta2* (n=59), cholangiocytes which were positive for expression *Sox9* and *Epcam* (n=130).

Analysis of expression signatures of tumor-infiltrating T cells which cataloged into 10 distinct subtypes including γδ T cells: exhausted T cells (Tex), naïve T cells (Tn) and interferon-stimulated genes (ISG) -positive T cells in both $CD4^+$ and $CD8^+$ compartments; the killer cell immunoglobulin-like receptor (KIR) -positive natural killer (NK) -like T cells in the $CD8^+$ compartment; [C-X-C motif chemokine receptor 5-positive ($CXCR5^+$) follicular helper T cells (Tfh), regulatory T cells (Treg), Th17 cells, and Th2 cells in the $CD4^+$ compartment.

For DEGs analysis, expression value of each gene in given cluster was compared against the rest of cells using Wilcoxon rank sum test. Significant upregulated genes were identified based on a set of stringent criteria. Genes were required to exhibit at least a 2-fold overexpression in

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the target cluster. Additionally, genes had to be expressed in more than 10% of the cells within the target cluster. Finally, only genes with a *P*-values less than 0.05 were considered significant. For Gene Set Enrichment Analysis (GSEA) pathway enrichment analysis, DEGs were initially identified and subsequently mapped to specific cellular processes and pathways. The calculated *P*-values were subjected to false discovery rate (FDR) correction, with an FDR threshold of ≤0.05 employed to determine significance. GSEA pathways meeting this criterion were defined as significantly enriched among the DEGs.

Tissue Microarray staining

We employed tissue microarray samples derived from early-stage HCC patients, encompassing both cancerous and adjacent non-cancerous tissues (HLivH180Su12, provided by Shanghai OutdoBiotech Co., Ltd.) to investigate the expression of CD36 and TFRC in $CD8^+$ T cells and their correlation with patient prognosis. Multiplex immunofluorescence staining and Prussian blue staining were performed as previously described. Subsequently, images were captured using an Akoya Vectra Polaris system and further analyzed with the inForm Automated Image Analysis Software (Akoya Biosciences) for cell detection and phenotyping.

Statistical analyses

All quantitative data were presented as mean±SEM or ±SD. For comparisons between two groups, a two-tailed unpaired *t*-test was performed. The Kaplan–Meier method was used to draw the survival curve, and the log-rank test method was used to analyze the statistical significance of the difference in survival time between the two groups. $P<0.05$ was considered statistically significant, as determined by statistical analysis using GraphPad Prism 10 (GraphPad Software, La Jolla, CA).

REFERENCE

1. Butler A, Hoffman P, Smibert P, Papalexi E, Satija R. Integrating single-cell transcriptomic data across different conditions, technologies, and species. Nat Biotechnol 2018;36:411-420.