Supplementary Table S1: Antibodies for flow cytometry

Antibodies	SOURCE	IDENTIFIER
Purified anti-mouse CD3ε Antibody	BioLegend	Cat#100302
Purified anti-mouse CD28 Antibody	BioLegend	Cat#102116
Biotin anti-mouse CD19 Antibody	BioLegend	Cat#115504
Streptavidin Nanobeads	BioLegend	Cat#480016
BV605 anti-mouse CD45 Antibody	BioLegend	Cat#103140
BV785 anti-mouse CD45 Antibody	BioLegend	Cat#103149
APC/Cyanine7 anti-mouse CD4 Antibody	BioLegend	Cat#100414
PE/Cyanine7 anti-mouse CD8a Antibody	BioLegend	Cat#100722
BV510 anti-mouse/human CD44 Antibody	BioLegend	Cat#103044
PE anti-mouse CD62L Antibody	BioLegend	Cat#104408
PerCP/Cyanine5.5 anti-mouse CD62L Antibody	BioLegend	Cat#104431
PE anti-mouse TNF-α Antibody	BioLegend	Cat#506306
APC anti-mouse IFN-γ Antibody	BioLegend	Cat#505809
PE/Cyanine7 anti-mouse IFN-γ Antibody	BioLegend	Cat#505825
BV785 anti-mouse CD69 Antibody	BioLegend	Cat#104543
AF700 anti-mouse CD103 Antibody	BioLegend	Cat#121441
PerCP/Cyanine5.5 anti-mouse CD103 Antibody	BioLegend	Cat#121415
APC anti-mouse Ki-67 Antibody	BioLegend	Cat#652405
FITC anti NWSHPQFEK Tag Antibody	Genscript	Cat#A01736
Biotin anti NWSHPQFEK Tag Antibody	Genscript	Cat#A01737
APC Streptavidin	BioLegend	Cat#405207
PE anti-mouse CD192 (CCR2) Antibody	BioLegend	Cat#150609
Bv785 anti-mouse CD366 (Tim-3) Antibody	BioLegend	Cat#119725
BV421 anti-mouse CD279 (PD-1) Antibody	BD Biosciences	Cat#565942
BV421 anti-human/mouse Granzyme B Antibody	BioLegend	Cat#396413
APC anti-human CA9 antibody	R&D Systems	Cat#FAB2188A
Recombinant human CA9 Protein (hFc Tag)	Sino Biological	Cat#10107-H02H
APC anti-human IgG Fc Antibody	BioLegend	Cat#410712
APC anti-human CD69 Antibody	BioLegend	Cat#310909
FITC anti-human CD62L Antibody	BioLegend	Cat#304804

Supplementary Table S2: Antibodies for CyTOF

Metal labels	Antibodies	SOURCE	IDENTIFIER
89Y	Purified anti-mouse CD45 (Maxpar® Ready) Antibody	BioLegend	Cat#103141
115In	Purified anti-mouse CD279 (Maxpar® Ready) Antibody	BioLegend	Cat#109113
141Pr	Purified anti-mouse CD103 Antibody	BioLegend	Cat#121402
142Nd	Purified anti-mouse CD197 (CCR7) Antibody	BioLegend	Cat#120101
143Nd	Purified anti-mouse CD19 (Maxpar® Ready) Antibody	BioLegend	Cat#115547
146Nd	Purified anti-mouse CD8a (Maxpar® Ready) Antibody	BioLegend	Cat#100755
150Nd	Purified anti-mouse CD127 (Maxpar® Ready) Antibody	BioLegend	Cat#135029
151Eu	Purified anti-mouse CD62L (Maxpar® Ready) Antibody	BioLegend	Cat#104443
152Sm	Purified anti-mouse CD69 (Maxpar® Ready) Antibody	BioLegend	Cat#104533
153Eu	Purified anti-mouse CD11b (Maxpar® Ready) Antibody	BioLegend	Cat#101249
154Sm	Purified anti-mouse CD3ε (Maxpar® Ready) Antibody	BioLegend	Cat#100345
160Gd	Purified anti-mouse CD49a Antibody	BioLegend	Cat#142602
165Ho	Purified anti-mouse CD186 (CXCR6) Antibody	BioLegend	Cat#151102
166Er	Purified anti-human/mouse CLA Antibody	BioLegend	Cat#321302
167Er	Purified anti-mouse CD11a Antibody	BioLegend	Cat#162902
168Er	Purified anti-mouse CD44 (Maxpar® Ready) Antibody	BioLegend	Cat#103051
169Tm	Purified anti-mouse CD183 (CXCR3) Antibody	BioLegend	Cat#126502
172Yb	Purified anti-mouse CD366 (Tim-3) Antibody	BioLegend	Cat#119702
173Yb	Purified anti-mouse CD223 (LAG-3) Antibody	BioLegend	Cat#125202
174Yb	Purified anti-mouse IFN-γ Antibody	BioLegend	Cat#505802
175Lu	Purified anti-human/mouse Granzyme B Antibody	BioLegend	Cat#372202
176Yb	Purified anti-mouse Ki-67 Antibody	BioLegend	Cat#652402
191Ir	Cell-ID Intercalator-Ir	Fluidigm	Cat#201192A
193Ir	Cell-ID Intercalator-Ir	Fluidigm	Cat#201192A
194Pt	Cell-ID™ Cisplatin	Fluidigm	Cat#201064
198Pt	Purified anti-mouse CD4 (Maxpar® Ready) Antibody	BioLegend	Cat#100561

Supplementary methods

B cell removal from murine primary lymphocytes

Briefly, the activated spleen cells were resuspended to 1x10^8/ml in medium with 10μg/ml biotin anti-mouse-CD19 antibodies and incubated at 4℃ for 30min, followed by centrifugation at 500g for 5 min and resuspension in medium with 20μl/ml MojoSort™ Streptavidin Nanobeads. After another round of incubation at 4℃ for 30min, the CD19+ B cells were labeled with Nanobeads and then removed by MojoSort™ Magnet (Cat#480019, biolegend).

Immunoblotting

The protocol for immunoblotting had been described previously.[1] Antibodies for immunoblotting were obtained from the following sources: anti-AKT (pan) (4685, Cell Signaling Technology, 1:1000), anti-Phospho-AKT (Ser473) (4060, Cell Signaling Technology, 1:2000), anti-GAPDH (AF5009, Beyotime, 1:1000), HRP goat anti-rabbit IgG (A0208, Beyotime, 1:2000), HRP goat anti-mouse IgG (A0216, Beyotime, 1:2000).

***In vitro* killing assay**

Approximately 5,000 target (KPC-hCA9 or Bxpc3-hCA9) cells were plated in each well of 96-well plates, followed by T cell addition at a ratio of 2:1, 1:1, 1:2, or zero. Fourteen hours later, target cells adhered to the surface of plates and T cells were resuspended and discarded. Then, 100 μl of medium with 10% Cell Counting Kit-8 (CCK-8; DOJINDO) was added into each well, followed by incubation at 37 °C for 1 hour. The OD450 was measured using a microplate reader. The lysis percentage was calculated as: lysis% = $100-(OD450_{\text{each well}}-OD450_{\text{blank}})/(OD450_{\text{zero}}-OD450_{\text{blank}})\times100$. Blank wells contained only CCK-8 medium.

Flow cytometry analysis and cell sorting

Tissues were digested and immune cells were enriched using 36%-Percoll gradient centrifugation.[2] Cells were blocked and then stained for cell surface epitopes.[2] Cells were permeabilized using a Foxp3/Transcription Factor Staining Buffer Set (ebioscience) before intracellular epitope staining. The antibodies are listed in Online Supplementary Table S1. Labeled cells were analyzed on a five-laser flow cytometer (Fortessa, BD) or sorted on a MoFlo Astrios EQ Cell Sorter (Beckman Coulter). Data were analyzed using FlowJo software (TreeStar). Data with few live cells were excluded.

Cytometry by time of flight (CyTOF) mass cytometry

Antibodies were mostly labelled with metals using Maxpar MCP9 antibody labelling kits (Fluidigm). The antibodies used are listed in Online Supplementary Table S2. Cells were labeled and then analyzed on a mass cytometer (Fluidigm, Helios). The cytofkit R package was used for t-distributed stochastic neighbor embedding (t-SNE) and PhenoGraph analysis.[3]

Cell staining for CyTOF

Resuspend 1–3 million cells in 200 µL Maxpar Cell Staining Buffer with 0.5uM Cell-ID™ Cisplatin and stain at room temperature (the same below) for 2min, followed by FcR blocking in 50 µL Maxpar Cell Staining Buffer for 10min. Add 50 µL of the surface marker antibody cocktail to each tube and stain for 30min, followed by washing with 500 µL Maxpar Cell Staining Buffer twice. Cells were permeabilized using a Foxp3/Transcription Factor Staining Buffer Set (ebioscience, cat#00-5523) before intracellular epitopes staining. Resuspend cells in 50 µL 1xPermeabilization with intracellular antibody cocktail and stain for 30min, followed by washing with 500 µL 1xPermeabilization Buffer twice. Resuspend cells in 200 µL Maxpar Fix and Perm Buffer with 125nM Cell-ID Intercalator-Ir and stain at 4 ℃ over night.

Bulk mRNA-seq

Total RNA was extracted from CAR-T cells using Trizol LS Reagent (Cat#10296010, Invitrogen). TruSeq strand-specific kit (Illumina) was used for mRNA-seq library preparation, followed by sequencing using

Illumina NovaSeq 6000 platforms. Differential expression analysis was performed using DESeq2 R package. ClusterProfiler R package was used to test the statistical enrichment of differential expression genes in KEGG pathways.

Plasmid construction of the 3rd generation human CAR

The 3rd generation human chimeric antigen receptor (hCAR) was pieced together using human CD8α signal peptide, anti-human-CAIX scFv, the hinge and transmembrane domains of human CD8α, the cytoplasmic domains of human CD28, 4-1BB, and CD3ζ.[4] The hCAR was cloned into the pLVX-EF1α-IRES-Puromycin lentiviral plasmid backbone (Cat#134665, Addgene) through EcoRI and MluI site to achieve overexpression. The human Runx3 cDNA was linked following hCAR through P2A self-cleaving peptide to achieve co-expression. Gene fragments were generated by chemical synthesis. The final plasmids were confirmed by Sanger sequencing.

Lentivirus production and human primary T cell transduction

Lentiviral supernatant was produced in the HEK293T cell line and concentrated by ultracentrifugation as previously described.[4] Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood of healthy donors using Human Lymphocyte Separation Medium (Solarbio) according to manufacturer's instructions. The PBMCs were activated with Dynabeads Human T-Activator CD3/CD28 (Gibco) and transduced with lentiviruses as previous described.[4] Transduced cells were resuspended after 4 hours and transferred to 12-well plates for expansion in the presence of 1μM AKTi-1/2 or vehicle. T cells were maintained in AIM-V Medium (Gibco) supplemented with 5% Immune Cell Serum Replacement (Gibco), 100IU/ml IL-2 and 0.05mM 2-mercaptoethanol.

reference

1. Zhang X, Huang X, Xu J, *et al.* NEK2 inhibition triggers anti-pancreatic cancer immunity by targeting PD-L1. *Nat Commun*. 2021;12(1):4536.
2. Sheng J, Zhang J, Wang L, *et al.* Topological analysis of hepatocellular carcinoma tumour microenvironment based on imaging mass cytometry reveals cellular neighbourhood regulated reversely by macrophages with different ontogeny. *Gut*. 2021.
3. Chen H, Lau MC, Wong MT, *et al.* Cytofkit: A Bioconductor Package for an Integrated Mass Cytometry Data Analysis Pipeline. *PLoS Comput Biol*. 2016;12(9):e1005112.
4. Cui J, Zhang Q, Song Q, *et al.* Targeting hypoxia downstream signaling protein, CAIX, for CAR T-cell therapy against glioblastoma. *Neuro Oncol*. 2019;21(11):1436-46.