Development of CAR-T cells specifically targeting cancer stem cell antigen DNAJB8 against solid tumors

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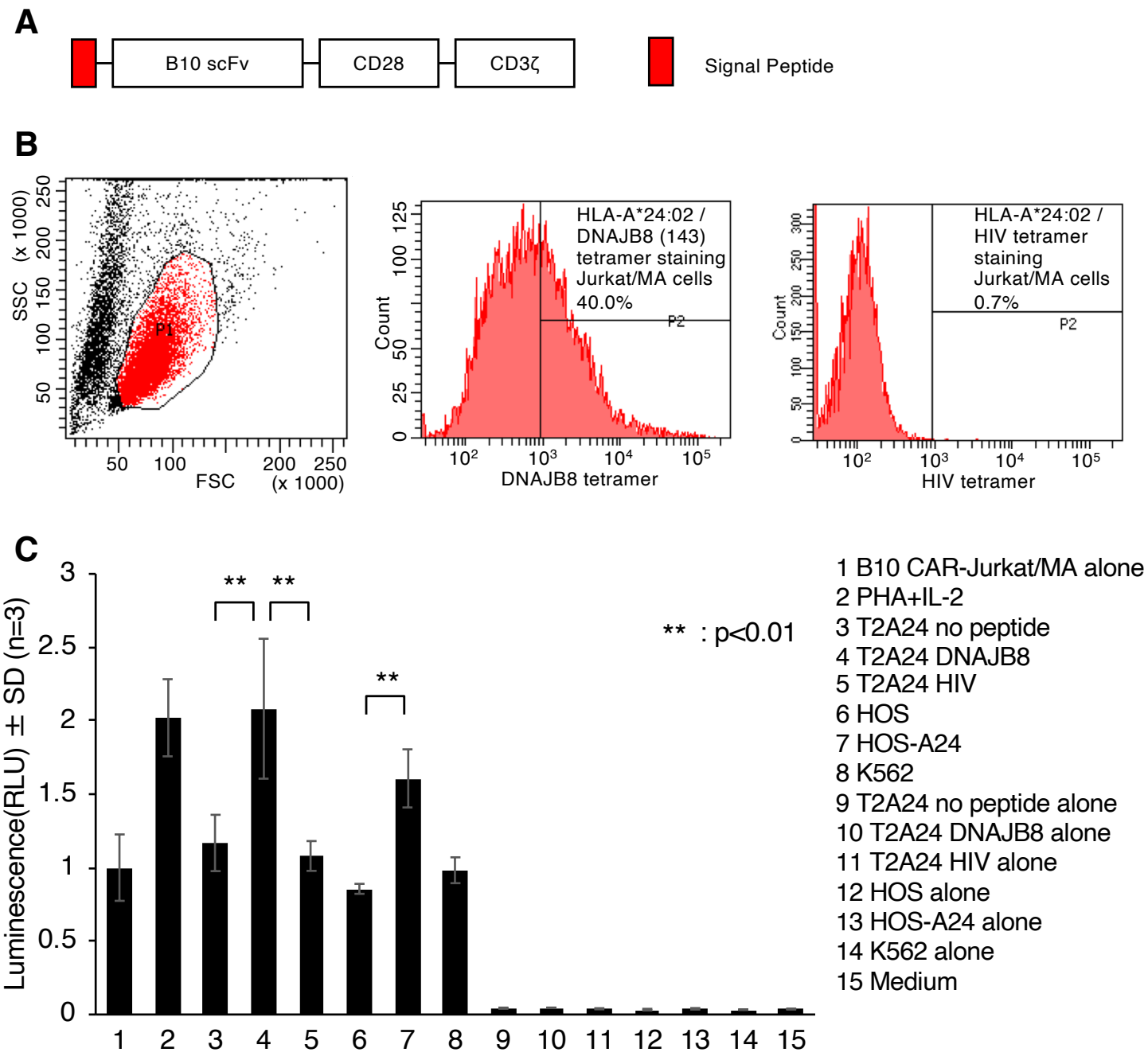
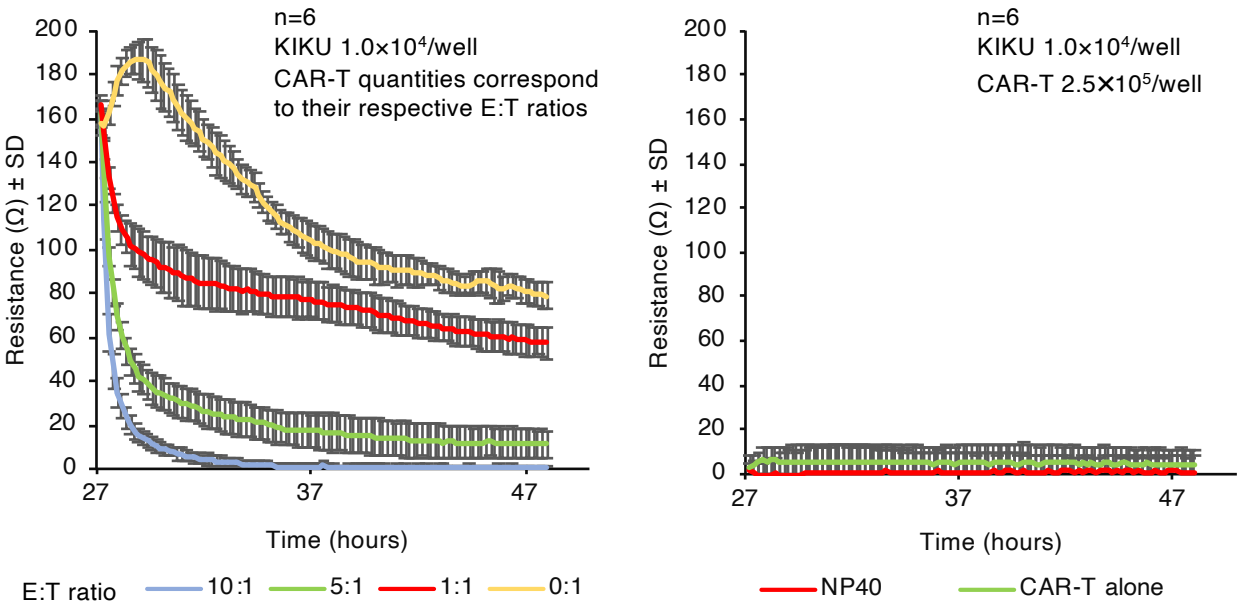
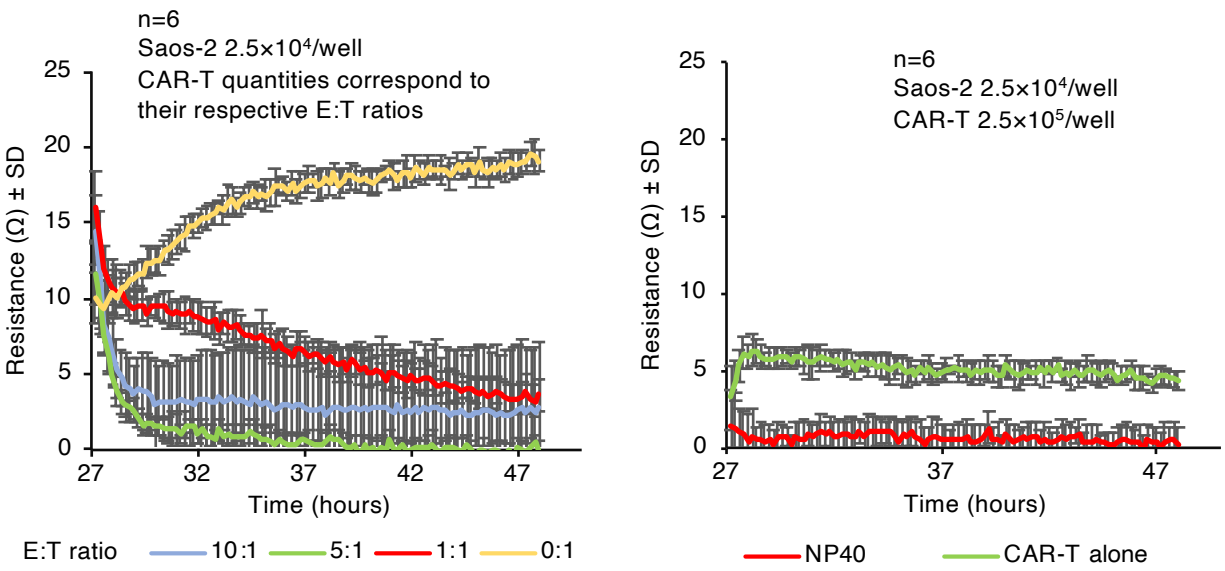


Figure S1. T cell activation assessed via the measurement of luciferase activity in the B10 CAR-transduced Jurkat/MA cell line. (A) Schema of the B10 CAR construct that does not contain ΔNGFR. (B) Flow cytometric plots of ΔNGFR⁻ B10 CAR-Jurkat/MA cells. Staining rates by HLA-A*24:02/DNAJB8_143 and HLA-A*24:02/HIV (negative control) peptide tetramers are shown. (C) NFAT (nuclear factor of activated T cells) signaling in the form of luminescence by luciferase produced by ΔNGFR⁻ B10 CAR-Jurkat/MA cells.

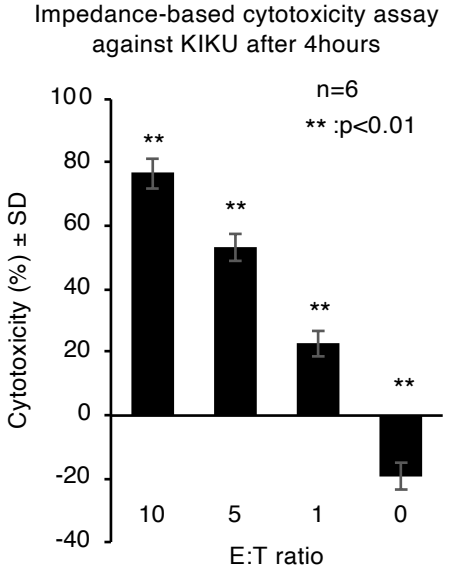
A


n=6
KIKU 1.0×10⁴/well
CAR-T quantities correspond to their respective E:T ratios
Resistance (Ω) ± SD
Time (hours)
E:T ratio
10:1
5:1
1:1
0:1
n=6
KIKU 1.0×10⁴/well
CAR-T 2.5×10⁵/well
NP40
CAR-T alone

B


n=6
Saos-2 2.5×10⁴/well
CAR-T quantities correspond to their respective E:T ratios
Resistance (Ω) ± SD
Time (hours)
E:T ratio
10:1
5:1
1:1
0:1
n=6
Saos-2 2.5×10⁴/well
CAR-T 2.5×10⁵/well
NP40
CAR-T alone

C


Impedance-based cytotoxicity assay against KIKU after 4hours
n=6
** :p<0.01
Cytotoxicity (%) ± SD
E:T ratio

D

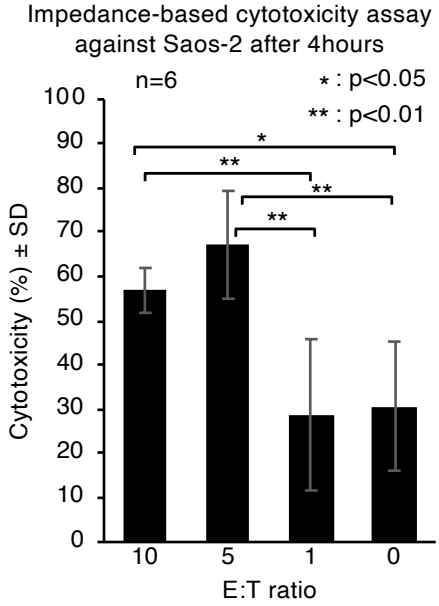

Impedance-based cytotoxicity assay against Saos-2 after 4hours
n=6
* : p<0.05
** : p<0.01
Cytotoxicity (%) ± SD
E:T ratio

Figure S2. Impedance-based assay also reveals the cytotoxicity of B10 CAR-T cells against both HLA-A*24:02- and DNAJB8-positive cell lines. The same B10 CAR-T cells expressing ΔNGFR described in the main text were used. The time at which the culture of target cells only was initiated is shown as time zero. At 24 h, B10 CAR-T cells were added in amounts corresponding to E:T ratios of 10:1, 5:1, and 1:1 to a fixed quantity of target cells. In the 0:1 ratio wells, only medium was added. (A, B) Resistance changes over time. (C, D) Calculated percent lysis at 28 h (4 h after the start of the co-culture). Target cell lines are indicated.

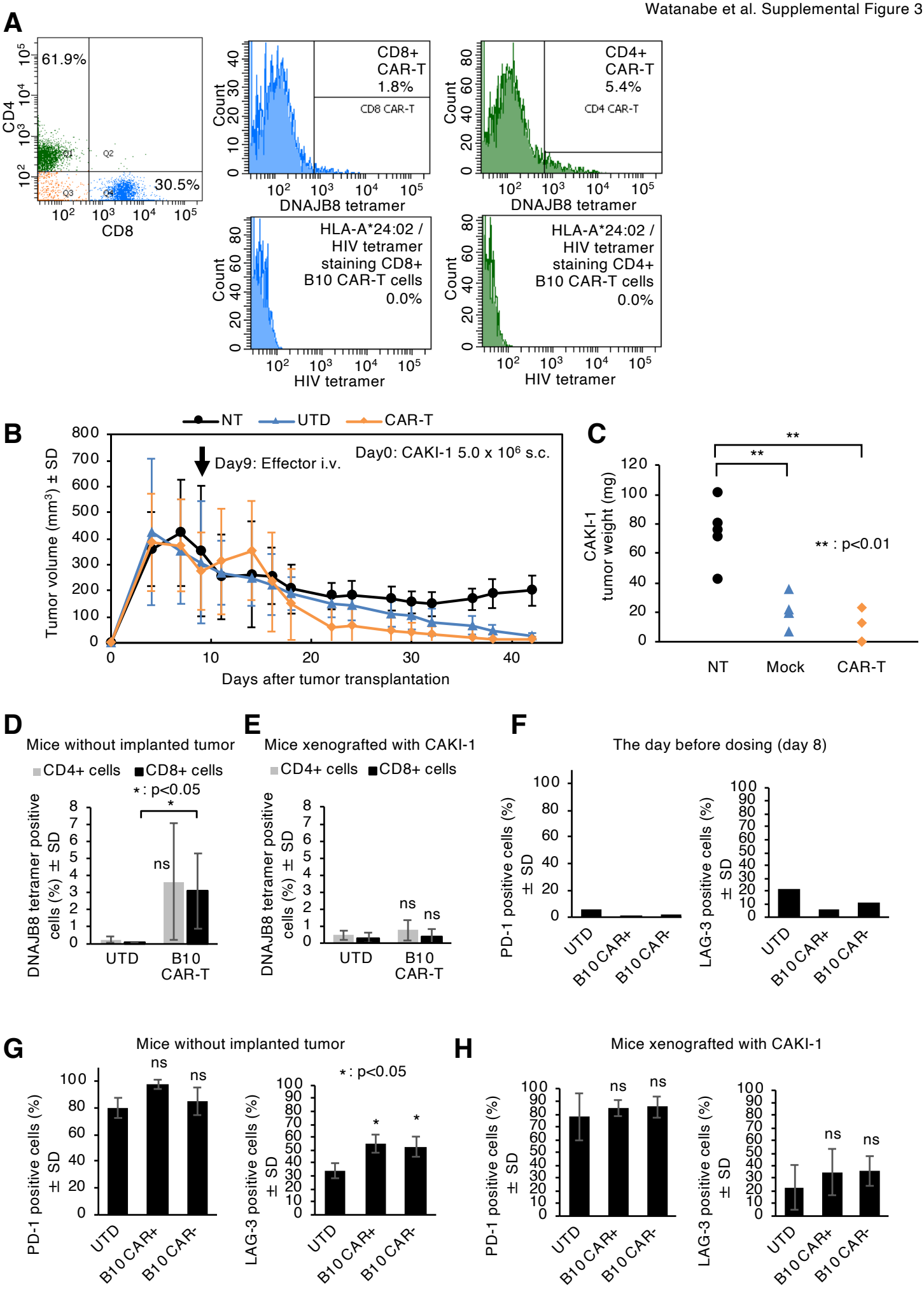
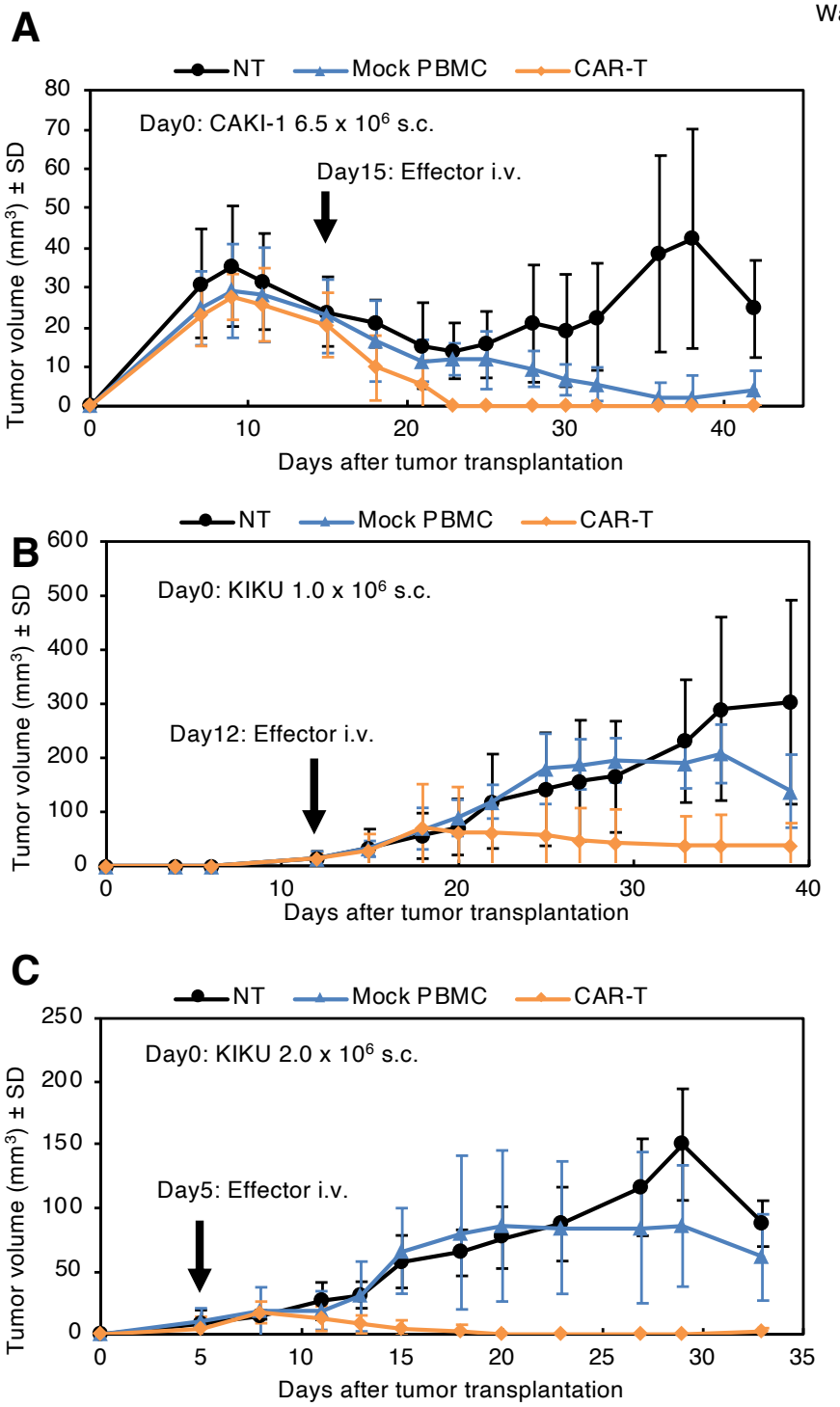

A
61.9%
30.5%
CD4
CD8
CD8+ CAR-T 1.8%
CD8 CAR-T
CD4+ CAR-T 5.4%
CD4 CAR-T
Count
DNAJB8 tetramer
HLA-A*24:02 / HIV tetramer staining CD8+ B10 CAR-T cells 0.0%
HLA-A*24:02 / HIV tetramer staining CD4+ B10 CAR-T cells 0.0%
HIV tetramer
B
NT
UTD
CAR-T
Day9: Effector i.v.
Day0: CAKI-1 5.0 x 10^6 s.c.
Tumor volume (mm^3) ± SD
Days after tumor transplantation
C
CAKI-1 tumor weight (mg)
** : p<0.01
NT
Mock
CAR-T
D
Mice without implanted tumor
CD4+ cells
CD8+ cells
* : p<0.05
DNAJB8 tetramer positive cells (%) ± SD
ns
UTD
B10 CAR-T
E
Mice xenografted with CAKI-1
F
The day before dosing (day 8)
PD-1 positive cells (%) ± SD
LAG-3 positive cells (%) ± SD
UTD
B10 CAR+
B10 CAR-
G
Mice without implanted tumor
H
Mice xenografted with CAKI-1

Figure S3. Effects of ΔNGFR⁻ B10 CAR-T cells in a xenografted mouse model. (A) Flow cytometric plots performed the day before dosing (on day 8). B10 CAR expression was defined as HLA-A*24:02/DNAJB8_143 tetramer (DNAJB8 tetramer) positive. CD8-positive cells and CD4-positive cells are colored blue and green, respectively. The reactivity of HLA-A*24:02/HIV peptide control tetramer against B10 CAR is also shown. Transduction efficiency was lower than in the main text because the activation and transduction protocols were not yet optimized. (B) Tumor volume (mean ± SD) over time in mice xenografted with CAKI-1 cells. (C) The weights of the removed tumors at the endpoint. (D, E) Percentage of DNABJ8 tetramer-positive cells in single-cell isolates from the spleen of the model mice that were positive for human CD45. (D) Mice without implanted tumor. (E) Mice xenografted with CAKI-1. (F–H) Percentage of cells expressing the exhaustion molecules PD-1 and LAG-3 among both human CD45- and human CD3-positive cells. (F) T cells with or without gene transduction. Analysis was performed the day before administration to mice. (G) Cells isolated from the spleen of mice without implanted tumor. (H) Cells isolated from the spleen of mice xenografted with CAKI-1. UTD: untransduced T cells before administration or single-cell isolation from the spleens of mice that received untransduced T cells. B10 CAR+: populations of transduced cells (or cells isolated from mouse spleen treated with transduced cells) stained with DNAJB8 tetramer. B10 CAR–: cell populations that were transduced or isolated from mice that received transduced cells.


A
NT
Mock PBMC
CAR-T
Day0: CAKI-1 6.5 x 10^6 s.c.
Day15: Effector i.v.
Tumor volume (mm3) ± SD
Days after tumor transplantation
B
Day0: KIKU 1.0 x 10^6 s.c.
Day12: Effector i.v.
C
Day0: KIKU 2.0 x 10^6 s.c.
Day5: Effector i.v.

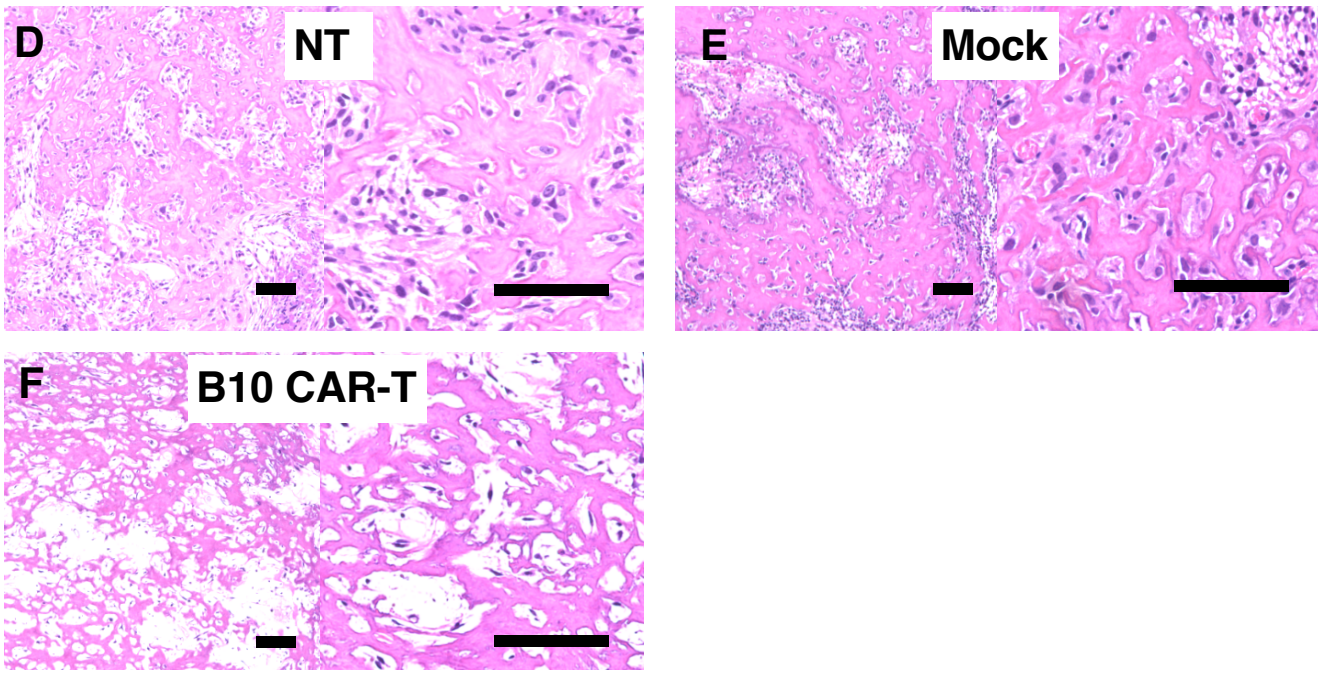

D
NT
E
Mock
F
B10 CAR-T

Figure S4. B10 CAR-T cells exert antitumor effects in a xenografted mouse model. (A–C) Changes in the average tumor volume shown in the mouse model in Figure 4. (A) Adoptive transfer therapy of B10 CAR-T cells into mice xenografted with CAKI-1 cells (CAKI-1 model). (B, C) Adoptive transfer therapy of B10 CAR-T cells into xenografted mice with KIKU cells. The B10 CAR-T cells were infused on day 12 (KIKU late-stage model, B) or day 5 (KIKU early-stage model, C). Tumor volume (mean ± SD) over time (NT group of panel B, n=10; the other group, n=5) are shown. (D–F) Hematoxylin and eosin staining of tumors harvested from mice in the KIKU late-stage treatment experiment. The histology of the NT (D), Mock (E), and B10 CAR-T (F) groups. Scale bar indicates 100 μm. NT: no treatment. Mock: treated by T cells transduced with a plasmid containing only ΔNGFR.

Supplemental Materials and Methods

Impedance-based cytotoxicity assay

The same B10 CAR-T cells expressing ΔNGFR described in the main text were used. The B10 CAR-T cells were co-cultured at E:T ratios of 1:1, 5:1, and 10:1 against a certain amount of the adherent target cell lines. KIKU cells and Saos-2 cells, each of which showed a sufficient increase in resistance in culture alone, were used as targets. After 24 h of target cell culture alone, the number of cells reaching a plateau in resistance was identified (1.0×10^4 cells for KIKU and 2.5×10^4 cells for Saos-2) and used. After 24 h of target cell culture alone, B10 CAR-T cells, only medium (E:T = 0:1, as a negative control), or detergent (NP-40, as a positive control) were added to the well. Resistance values were also measured in wells to which 2.5×10^5 B10 CAR-T cells were added at 24 h to wells containing only medium and no target. The resistance was measured in real-time using the Maestro Z instrument (Axion BioSystems, Atlanta, GA). In addition, the percent lysis was calculated from the resistance value at 28 h (4 h after the start of the co-culture) using the following equation:

Percent lysis = [1 – (Resistance of each well / Average resistance of all wells containing target cells at 24 h)] × 100

Generation of another B10 CAR construct that does not contain ΔNGFR using a different retroviral vector

Prior to creating the B10 CAR construct containing ΔNGFR described in the main text, the same second-generation B10 CAR gene containing neither ΔNGFR nor P2A junction was incorporated into another type of retroviral vector. This structure was only used within supplemental experiments.

B10 CAR transduction using retroviral vector without ΔNGFR

The human T cells isolated by the methods described in the main text from the whole blood of HLA-A24-positive healthy donors were transduced. Half of the T cells were cultured in the same way without gene transfer (untransduced T cells). ΔNGFR$^-$ B10 CAR-T cells produced in a timely manner were not frozen and were used in the assay and for administration to mice. Before experiments, they were cultured in 10% human serum containing AIM-V medium without any stimulants for at least 48 h.

Cell lines and culture

An NFAT-luciferase reporter gene-transduced Jurkat/MA cell line was kindly donated by Dr. Yoshinobu Ichiki (University of Occupational and Environmental Health, Japan) and Dr. Sjoerd H. van der Burg (Leiden University Medical Centre, the Netherlands). This cell line was transduced with retroviral vector without ΔNGFR (ΔNGFR$^-$ B10 CAR-Jurkat/MA). B10 CAR-Jurkat/MA cells were maintained in RPMI 1640 (Sigma-Aldrich) supplemented with 10% FBS.

Flow cytometric analysis of CAR expression on T cells or Jurkat/MA cells

To evaluate the frequency of CAR expression, the following antibodies and tetramers were used: PE anti-hCD8 (BD Biosciences, Cat. #340046, RRID: AB_400005), FITC anti-hCD4 (BD Biosciences, Cat. #340133, RRID: AB_400007), and PE-Cy7 anti-hCD3 (BD Biosciences, Cat. #341091, RRID: AB_400215). The CAR-T cells or CAR-Jurkat/MA cells were stained with one of the following fluorescent-labeled HLA class I tetramers: HLA-A*24:02 DNAJB8_143 tetramer APC (MBL, T-select TSCM-1TA),

HLA-A*24:02 DNAJB8_143 tetramer PE (MBL, T-select TSCM-1), HLA-A*24:02 HIV env tetramer-RYLRDQQLL FITC (MBL, T-select TS-M007-3), and HLA-A*24:02 HIV env tetramer-RYLRDQQLL PE (MBL, T-select TS-M007-1). Dead cells were stained with the AmCyan Fixable Aqua Dead Cell Stain Kit (Thermo Fisher Scientific, Cat. #L34965) and discriminated. The stained cells were analyzed with a FACSCanto II (BD Biosciences, RRID: SCR_018056).

Luciferase reporter assay

B10 CAR-Jurkat/MA cells (2.0×10^5) were co-cultured with the target cell lines (2.0×10^4) for 18 h at 37°C. As a positive control, wells with 10 μg/mL of phytohemagglutinin-P (Fujifilm Wako Pure Chemical Corporation, Japan, Cat. #161-15251) and IL-2 (100 IU/mL) added to the B10 CAR-Jurkat/MA cells were prepared. After the incubation, the Bright-Glo Luciferase Assay System (Promega, Madison, WI, Cat. #E2620) was used according to the manufacturer's protocol and the results were analyzed with an Infinite M1000 Pro multiplate reader (Tecan). Measured luminescence values were standardized by considering the readings for the well of B10 CAR-Jurkat/MA cells alone as 1.

Mouse models that were given ΔNGFR⁻ B10 CAR-T cells

A total of 25 male NSG mice that were 20 to 32 weeks old were purchased. Fifteen mice received subcutaneous injections of 5.0×10^6 CAKI-1 cells in 100 μL of Matrigel (Corning, NY, Cat. #356234) into the back. Nine days after the tumor injection, the mice were divided into three groups (five mice each) according to the average tumor size. One group received an intravenous infusion of produced B10 CAR-T cells, another group received an intravenous administration of untransduced T cells, and the remaining group

did not receive any cells. At the same time, of the remaining 10 mice without implanted tumors, 5 received B10 CAR-T cells into a vein and 5 were given intravenous infusions of untransduced T cells. Tumor volume was assessed three times a week using a caliper and calculated. After the endpoint, the spleens were removed and analyzed in the following way.

Isolation of human T cells from the mouse spleen after intravenous administration

Each dissected spleen was chopped into small pieces with sterile scissors, mixed with 2.2 mL of RPMI1640 (Sigma-Aldrich), and placed in a gentleMACS C Tube (Miltenyi Biotec, Germany, Cat. #130-093-237, RRID: SCR_020270). The tubes were then placed in the gentleMACS™ Octo Dissociator with Heaters (Miltenyi Biotec, Cat. #130-096-427, RRID: SCR_020271) and processed according to the instructions. The separated cell solution was passed through a cell strainer (Corning, Cat. #352360) and lysed with BD Pharm Lyse Lysing Buffer (BD Biosciences, Cat. #555899) to lyse the erythrocytes.

Flow cytometric analysis of the expression of the exhaustion molecules PD-1 and LAG-3 in CAR-positive and -negative cells separated from mouse spleens

In addition to the above-described CAR staining, the following antibodies were used: APC/Cyanine7 anti-hCD45 (BioLegend, Cat. #368516, RRID: AB_2566376), FITC anti-hCD279 (PD-1) (BioLegend, Cat. #329903, RRID: AB_940477), and PE anti-hCD233 (LAG-3) (BioLegend, Cat. #369306, RRID: AB_2629592). The stained cells were analyzed with a FACSCanto II. Populations of cells negative for dead cell staining and positive for human CD45 staining were gated and analyzed for positive and negative CAR tetramer staining. Separate stained samples were used for staining to evaluate the

expression of PD-1 and LAG-3 molecules, and positive thresholds were established using samples that were not stained for these antibodies only (fluorescence minus one control).