SUPPLEMENTARY MATERIALS

Supplementary Methods

Cell lines and primary cells

786-O (CRL-1932), ACHN (CRL-1611), REH (CRL-8286, HEK293T (CRL-3216), and Jurkat (TB-152) cell lines were acquired from ATCC. No authentication was performed and all cells were purchased directly from the source. No misidentified cell lines were used. All cell lines were tested for mycoplasma and were negative. CD70 knockout Jurkat cells were generated using CRISPR/Cas9 gRNA. Dissociated tumor cells were obtained from Conversant Bio. Primary T cells were isolated from human peripheral blood mononuclear cells using human Pan T cells isolation kit (StemCell Technologies). T cells were activated using human TransAct (Miltenyi Biotec) and cultured in the presence of IL-2.

Immunohistochemistry staining of RCC subtypes

Unstained sections of human renal cell carcinoma tissue (RCC) microarrays (US Biomax) were deparaffinized, hydrated, and subjected to antigen retrieval using Diva Decloaker (1X, Biocare Medical). Sections were blocked with 5% normal horse serum (Vector Labs) for 1 hour at room temperature followed by incubation with an anti-CD70 mouse monoclonal (clone 301731, 1:250, R&D Systems) or IgG2B isotype control (1:250, R&D Systems) antibody at 4 ºC overnight. Slides were then stained with a peroxidase polymer anti-mouse Ig reagent (Vector Labs) for 30 min. Liquid DAB + substrate reagent (Dako) was added to the slides to enable chromogenic detection. The slides were then counterstained with hematoxylin, dehydrated, and cover slipped. CD70 staining was reviewed and scored by a board-certified pathologist. Images were collected at the indicted magnifications using a Keyence BZ-X710 microscope.

CAR transduction and characterization in Jurkat and primary T cells

Plasmids to produce lentiviral vectors (pLVX-EF1α-IRES-Puro, pLVX-EF1α- TurboGFP-P2A, pLVX-EF1α-TurboBFP-P2A, pMD.2G, psPAX2) were sourced from Clontech, Mountain View, CA and École Polytechnique Fédérale de Lausanne, Lausanne, France. DNA synthesis, molecular cloning procedures, and plasmid preparations were conducted by Genscript, Piscataway, NJ. The nucleotide sequences encoding CD70 CARs were synthesized and used to replace the IRES-Puro cassette in the pLVX-EF1α-IRES-puro plasmid to generate various CAR constructs. CAR constructs with GFP or BFP tags were generated by adding nucleotide sequences downstream of P2A in the pLVX-EF1α- TurboGFP-P2A or pLVX-EF1α-TurboBFP-P2A plasmid, respectively. HEK293T cells were resuspended in complete DMEM medium and plated in 6-well plates. The following day, cells were transfected with the CAR construct along with psPAX2 and pMD.2G plasmids at a ratio of 1:3:1, respectively, using Lipofectamine2000 according to the manufacturer's manual. Forty-eight hours after transfection, the lentiviral vector (LVV)-containing supernatant was harvested, filtered through a 0.45 μm syringe filter into a fresh 50 mL conical tube and immediately used for transduction. Jurkat cells were obtained from ATCC and isogenic CD70-negative cells (CD70 KO) were generated using CRISPR/Cas9 gRNA targeting *CD70*. Wildtype Jurkat (CD70 WT) or CD70 KO Jurkat cells were activated using human TransAct using manufacturer's protocol and 48h later transduced with 50% LVV supernatant (v/v). Four days post-transduction, cells were stained with anti-CD69 antibody for 20 min at 4°C and acquired on the flow cytometer to determine their activation status. Primary T cells isolated and frozen as described previously, were thawed and activated using human TransAct using manufacturer's protocol and 48h later transduced with 50% LVV supernatant (v/v). Where T cell receptor-α (TRAC), CD52, or CD70 knockout T cells were used, 6 days after activation, T cells were transfected with 50 μg/mL per TALEN® mRNA (Cellectis & TriLink Biotechnologies) using

AgilePulse MAX electroporators (BTX Harvard Apparatus). Nine days after activation, cells were stained with anti-CD70 (clone 41D12), anti-CD25, and anti-4-1BB antibodies 20 min at 4°C and acquired on the flow cytometer. CD70 expression and activation status of CAR^+ cells were identified by gating on BFP. Fourteen days after activation, cells were stained with anti-CD62L and anti-CD45RO antibodies 20 min at 4°C and acquired on the flow cytometer. Memory phenotype of CAR^+ cells were identified by gating on BFP. Where TRAC, CD52, or CD70 knockout T cells were used, cells were stained with anti-TCRαβ, anti-CD52, or anti-CD70 antibodies fourteen (or nineteen for large-scale process) days after activation to determine knockout efficiencies. Recombinant human IL-2 was added throughout T cell culture and TCRαβ-positive cells were depleted using TCRαβ isolation kit (Miltenyi Biotec) at the end of process. T cells were cryopreserved in 90% FBS/10% DMSO using rate-controlled freezing chambers and stored in liquid nitrogen vapor phase. All functional assays were performed with cells after recovery from cryopreservation.

Flow cytometry and antibody list

Cells were resuspended in the Grex plate and 50-100 uL cell suspension was transferred to a 96-well U-bottom plate. Cells were washed with 150 uL FACS buffer (PBS + 2% FBS + 2 mM EDTA) and centrifuged for 2 minutes at 400 × *g*. Cells were suspended in 100 µL of FACS buffer containing appropriate cocktail of antibodies, stained for 20 minutes in the dark at 4°C and washed with 150 µL of FACS buffer. After the wash step, cells were suspended in 150 µL of FACS buffer for flow cytometry analysis. Samples were acquired using a Cytoflex flow cytometer (Beckman Coulter) and the data was analyzed using FlowJo version 10.6.0.

Reagent Name	Supplier/Source	Catalog Number/Clone	Concentration/uL per test
BUV395 Anti-CD3	BD Horizon	563546	3 uL/test

BV786 Anti-CD4	BioLegend	317442	3 uL/test
BV510 Anti-CD8	BioLegend	301048	1 uL/test
BV605 Anti-CD62L	BioLegend	304834	2 uL/test
APC-Cy7 Anti-CD45RO	BioLegend	304228	2 uL/test
APC Anti-CD70	Generated in-house	Clone 41D12	10 ug/mL
PE-Cy7 Anti-CD69	BioLegend	310912	2 uL/test
BV421 Anti-CD25	BioLegend	302630	2 uL/test
APC Anti-CD137 (4-1BB)	BioLegend	309810	2 uL/test
PE rituximab (RTX)	Generated in-house	Rituximab	10 ug/mL
FITC Anti-CD52	BioLegend	316004	5 uL/test
PE Anti-TCRαβ	Miltenyi	130-113-531	2 uL/test

In vitro cytotoxicity assays

786-O, ACHN, and REH cell lines were transduced with lentiviral vector (LVV) made in house using a construct for co-expression of luciferase and nuclear-localized GFP bearing resistance to Blasticidin. Luciferase-expressing target cells were added to 96-well white-wall plates (and allowed to attach overnight – if adherent), then co-cultured with CAR+ cells at defined E:T ratios for 72 hours in 200 µL of target cell culture medium without IL-2. After 72 hours, cell culture supernatant was removed, ONE-Glo reagent was added and luminescence was acquired on SpectraMax luminometer. Luminescence background was assessed in wells containing only T cells or media and was found to be negligible. Relative luminescence units (RLU) were converted to percentage of lysed target cells using the formula 100 × [1- (RLUtest/RLUcontrol)]. Untreated target cells were used to determine RLU control. Short-term cytotoxicity was determined by measuring the reduction of luminescence signal from live target cells after 72 hour co-culture with CAR T cells at increasing effector to target (E:T) ratios. Luciferase-expressing 786-O, ACHN, or REH target cells were added to 96-well white-wall plates (and allowed to attach overnight – if adherent), then co-cultured with CAR+ cells at a predetermined E:T ratio of 3:1, 10:1, or 1:5, respectively for 72 hours in 200 µL of target cell culture medium without IL-2. After 3 days, cells were thoroughly mixed using Viaflo 96 (Integra Biosciences) and 100 µL cell suspension was

transferred to a new 96-well plate containing target cells. The spent plate was read out by adding 100 µL ONE-Glo reagent and luminescence was acquired on Spectramax luminometer. Luminescence background was assessed in wells containing only T cells or media and was found to be negligible. Relative luminescence units (RLU) were converted to percentage of lysed target cells using the formula 100 × [1- (RLUtest/RLUcontrol)]. Untreated target cells were used to determine RLU control. This process was repeated for 10 rounds over 5 weeks, i.e., cells were transferred to a new plate containing targets every 3-4 days. Long-term cytotoxicity was determined by measuring the reduction of luminescence signal from live target cells after repeated co-culture with CAR T cells every 3-4 days.

Kinetics and binning biosensor assays

All SPR analysis of CD70-CD3 bispecific antibody, anti-CD70 Fabs or scFvs and CD70 antigen single point mutation supernatant samples were determined on a Biacore T200 SPR instrument (Cytiva, Marlborough, MA). For kinetic analysis of CD70-CD3 bispecific antibody, surfaces capable of capturing human and cyno CD70 Avi-tagged proteins were prepared by amine-coupling of an anti-Avi reagent (anti-Avi antibody, Genscript, Catalog #A00674) to a Biacore Series S CM4 sensor chip using a running buffer of 10mM HEPES, 150mM NaCl, 0,05% (v/v) Tween-20, pH 7.4 at 25°C. All surfaces of the sensor chip were activated with a 1:1 (v/v) mixture of 400mM 1-Ethyl-3-(3-Dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 100mM N-hydroxysuccinimide (NHS) for 7 min at 10 µL/min. The anti-Avi reagent was diluted to 30µg/mL in 10mM sodium acetate (pH 4.5) and injected on all four flow cells for 7 min at 10 µL/min, then all flow cells were blocked with 100mM ethylenediamine in 200mM Borate buffer pH 8.5 for 7 min at 10µL/mL. For kinetic analysis anti-CD70 Fabs or scFvs and CD70 antigen single point mutation supernatant samples and premix binning of anti-CD70 Fabs, Biotin

CAPture kit (containing CAP chips, Biotin CAPture and regeneration reagents) was purchased from Cytiva (Marlborough, MA) and used according to the manufacturer protocol.

All interaction experiments were performed at 37°C using the same running buffer as described above supplemented with 1 mg/mL BSA. The affinities for the interactions of CD70-CD3 bispecific antibody were determined by capturing 5µg/mL of both human and cynomolgus recombinant CD70 proteins on the anti-Avi chip surface, then buffer and the bispecific antibody at concentrations of 0.16, 0.8, 4, 20 and 100nM were injected for 2 min at 30µL/min. The dissociation was monitored for 5 min followed by regeneration of all flow cells with three 30-second injections of 75mM phosphoric acid. To measure the binding kinetics of anti-CD70 Fabs or scFvs against hCD70, 0.5 µg/mL of site-specifically biotinylated hCD70 (bt-hCD70) at the Avi tag was captured for 2 minutes at 10µL/min on Biacore's regeneratable streptavidin capture (CAP) surface. Then, all flow cells were then blocked with 20µM Amine-PEG2-Biotin (APB). Buffer and all anti-CD70 Fabs or scFvs at concentrations of 1.2, 3.7, 11.1, 33.3, and 100 nM were injected as analytes for 2 minutes and dissociation was monitored for 15 minutes at 30µL/min. The surface was regenerated according to the manufacturer protocol. All interactions were measured in triplicate using three independent analyte dilution series and captured samples. All sensorgrams were double-referenced with the data from the buffer analyte injections (Myszka, 1999). Data was fitted to a 1:1 Langmuir binding model with mass transport using Biacore T200 Evaluation Software (version 2.0).

All CD70 antigen single point mutation samples were site-specifically biotinylated via co-expression with BirA, followed by dialysis and filter prior to SPR analysis. All these biotinylated CD70 samples were captured as neat supernatants on the Biacore's CAP surface. All flow cells were then blocked with 20µM Amine-PEG2-Biotin (APB). Buffer and 100nM of all eight anti-

CD70 Fabs were injected as analytes for 2 minutes and dissociation was monitored for 15 minutes at 30µL/min. Surface regeneration and data analysis was the same as the kinetic analysis of anti-CD70 Fabs as described above.

To perform premix binning of anti-CD70 Fabs, all anti-CD70 Fab clones were biotinylated using 1:1 biotin:protein ratio. 5 or 10µg/mL of each biotinylated anti-CD70 Fab clone was captured for 2 minutes at 10µL/min on Biacore's regeneratable CAP surface. All flow cells were then blocked with 20µM APB. Buffer, 100nM human CD70 (hCD70) binding sites, or the premix of 100nM hCD70 binding sites and each anti-CD70 Fab at 1µM (only CAR 24 at 5µM due to weaker affinity and faster off-rate) as premix analyte was then injected in all flow cells for 2 min and dissociation was monitored for 5 minutes at 30µL/min. When a premixed analyte sample showed a lower or similar binding response compared to hCD70 alone, i.e. an "intermediate" response, a secondary detection method was used to disambiguate the result. Human IgG format of anti-CD70 clone was used as the premixed analyte in the follow-up assay. Biotinylated anti-CD70 Fab was captured as described above followed by sequential injections of the premix of hCD70/anti-CD70 hIgG at concentrations as stated above and anti-human Fc (Southern Biotech, Cat. No. 2014-01) at 100nM were injected for 2 minutes at 30µL/min using dual inject. Anti-human Fc was specific to the premixed anti-CD70 hIgG and used as a secondary detection reagent to confirm whether the "intermediate" response was due to a sandwiching interaction between the premixed and captured anti-CD70 clone.

Renal cell carcinoma xenograft models

All animal studies were performed by trained staff at Allogene Therapeutics South San Francisco under approval by the Allogene Therapeutics Institutional Animal Care and Use Committee (IACUC), and all applicable animal care and use regulations, guidelines, and policies were

followed. Female NOD-scid IL-2Rγnull (NSG) 8-12-week-old mice (Jackson Laboratory) were used for all experiments. 786-O cells were thawed and cultured at 37°C in RPMI containing 10% FBS. After 2-3 passages, cells were harvested using Accutase and suspended at 50×10^6 cells/mL in serum-free RPMI. The cells were implanted subcutaneously on the right flank of the mouse using 100 µL cell suspension mixed with 100 µL Matrigel for a final dose of 5×10^6 cells/mouse. Tumor volume and body weight measurements were performed twice weekly using digital calipers (Mitutoyo). Tumor volumes were calculated by dividing the tumor length by 2 and multiplying with the square of the width as measured using Mitutoyo digital calipers. Animals were matched by tumor volume and randomly assigned into treatment groups of 5-10 animals, 14 days after implantation, at a tumor volume of ~ $200mm^3$.

ACHN-nucLucGFP cells were injected as a 200 µL bolus intravenously (i.v.) via tail vein for a dose of 1×10^6 cells/mouse. Bioluminescence and body weight measurements were performed twice weekly using the IVIS Spectrum (Perkin Elmer). Bioluminescence imaging was performed at least 9 minutes after animals were injected with 3 µg D-Luciferin in 200 µL PBS intraperitoneally (i.p.). The total animal body area from the base of the tail to the tip of the nose was used for analysis and background subtraction was performed using average pixel intensities of a background reference field in the same image. Animals were matched by luminescence and randomly assigned into treatment groups of 5-10 animals, 14 days after implantation.

NSG mice bearing patient derived RCC xenograft tumors on the right flank were purchased from Jackson Laboratory. Tumor volume and body weight measurements were performed twice weekly using digital calipers (Mitutoyo). Tumor volumes were calculated by dividing the tumor length by 2 and multiplying with the square of the width as measured using Mitutoyo digital calipers. Animals were matched by tumor volume and randomly assigned into treatment groups of 8

animals, 21 days after implantation, at a tumor volume of ~250mm^3. CAR T cells were generated as previously described. For all batches of CAR T cells, cryopreserved non-transduced control T cells were thawed, washed into RPMI and mixed in order to maintain the same number of total T cells in each treatment group. CAR T cells were given at various doses as a 200 µL bolus via tail vein injection. Mice in the ACHN study were given DietGel Boost supplement once a week to prevent transient body weight loss specific to this model. Animals were euthanized when they exhibited disease model-specific endpoints such as tumor volume of 2250 mm^3 or body weight loss exceeding 20%.

Tissue Cross Reactivity (TCR) assay

The binding domains of CARs 3, 17, 23, along with an isotype control were generated in soluble form and screened for binding at an optimal concentration of 0.5 µg/mL and 4x-optimal concentration of 2 µg/mL against a panel of 36 normal tissues by immunohistochemistry. Cell pellets from CD70-positive and CD70-negative cells lines were included as controls. The slides were rinsed with Tris buffered saline, 0.15M NaCl, pH 7.6 (TBS), endogenous peroxidase was quenched, incubated with avidin solution, incubated with biotin solution, and treated with a protein block designed to reduce nonspecific binding. Following the protein block, precomplexed primary (test or control article) and secondary antibodies were applied to the slides for 2 hours. Next, the slides were rinsed twice with TBS, treated with the ABC Elite reagent for 30 minutes, rinsed twice with TBS, and then treated with ImmPACT DAB for 4 minutes as a substrate for the peroxidase reaction. All slides were rinsed with tap water, counterstained, dehydrated, and mounted. TBS + 1% BSA served as the diluent for all antibodies and ABC reagent.