**Multiple injections of electroporated autologous T cells expressing a chimeric antigen receptor mediate regression of human disseminated tumor**

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## Supplementary Methods

**Construction of in vitro transcription (IVT) mRNA vectors for CARs.** Mesothelin (ss1) and CD19 specific CARs (1, 2) were PCR amplified using the primers ss1F (cctaagcttaccgccatggccttaccagtgac ), CD19F (cctaagcttaccgccatggccttaccagtgaccgcc ) and zetaR (cctgcggccgc ttagcgagggggcagggcc ). The PCR products were subcloned into pGEM.64A based vector by replacing GFP of pGEM-GFP.64A (3) to produce pGEM.64A based ss1 and CD19 vectors. To add 5' or 3' un-translational regions (UTR) and longer poly(A) to the constructs, the 64 poly(A) sequence in pGEM.ss1.bbz.64A or pGEM-CD19.bbz.64A vectors was replaced by two repeats of 3' UTR from beta globulin (2bgUTR) with or without 150 poly(A) sequences (150A) synthesized by PCR and further confirmed by sequencing. However, pGEM based vectors use ampicillin for selection, and this is not compatible with FDA regulatory guidance for GMP production and later clinical application. Thus, the CAR cDNA with UTRs were transferred to pDrive (Qiagen), which also uses kanamycin for selection. First, ss1.bbz.2bgUTR.150A or CD19.bbz.2bgUTR.150A was cut from the pGEM vector by Hind III and NdeI digestion (fill-in blunt end) and subcloned into pDrive by KpnI and NotI (fill-in blunt end). The insert with correct orientation was confirmed by sequencing to generate pDrive.ss1.bbz.2bgUTR.150A and pDrive.CD19.bbz.2bgUTR.150A. There were two steps to

finalize the vectors for potential clinical use: 1) The ampicillin resistance gene in the pDrive vectors was deleted by double digestion with AhdI and BciVI; 2) Internal open reading frames in both CD19.bbz and ss1.bbz were deleted by mutagenesis PCR to produce pD-A.19.OF.2bg.150A and pD-A.ss1.OF.2bg.150A.

**RNA in vitro transcription (IVT).** Three RNA IVT systems were used to compare RNA quality, quantity and cost: mMESSAGE mMACHINE® T7 Kit (Ambion, Inc) that uses the regular cap (RC) analog 7-methylGpppG; mMESSAGE mMACHINE® T7 Ultra (Ambion, Inc) that generates IVT RNA with Anti-Reverse Cap Analog (ARCA, 7-methyl(3'-O-methyl)GpppG)m7G(5')ppp(5')G), and the mScript™ RNA System (Epicentre, Madison, WI) that uses capping enzyme (CE) and 2'-O-Methyltransferase capping enzyme to generate Cap 1 IVT RNA (Epicentre). The RC is incorporated with a capping efficiency of up to 40%, while the ARCA increases capping efficacy up to 80% and the CE system can result in up to 100% capping efficiency. The various IVT RNA products were purified using an RNeasy Mini Kit (Qiagen, Inc., Valencia, CA) and purified RNA was eluted in RNase-free water at 1–2 mg/ml.

**Preparation of clinical grade IVT RNA.** To generate regulatory compliant plasmid DNA vectors containing the CAR open reading frames (ORF) without internal ORFs, DNA inserts for CAR cDNA with UTR and poly-A sequences were subcloned from the pGEM based vectors to pDrive vector that contains a kanamycin selection marker to generate pdrive.19bbz (for CD19-bbz) and pDrive.ss1bbz (for ss1-bbz) as described above. To eliminate potential aberrant proteins translated from internal ORFs nested inside the CAR ORF, all internal ORF in both CD19-bbz and ss1-bbz larger than 60 bp in size were mutated by mutagenesis PCR. Thus pD-A.19.OF and pD-A.ss1.OF that are free of internal ORFs were generated for 19-bbz and ss1-bbz respectively.

**RNA electroporation of T cells.** On day 10 of culture, the activated T cells were collected and electroporated. Two electroporation systems were used: BTX CM830 (Harvard Apparatus BTX, Holliston, MA, USA), and Maxcyte (Maxcyte Inc, Rockville, MD, USA). For electroporation using BTX EM830, the stimulated T cells subjected to electroporation were washed three times with OPTI-MEM (Invitrogen) and resuspended in OPTI-MEM at the final concentration of 1-3x$10^8$/ml. Subsequently, 0.1 to 0.2 ml of the cells was mixed with 10ug/0.1ml T cells of IVT RNA (or as indicated) and electroporated in a 2-mm cuvette (Harvard Apparatus BTX, Holliston, MA, USA). For electroporation using Maxcyte, the instruction manual was followed using OC-400 processing chamber (Maxcyte Inc, Rockville, MD, USA) with integrated programs.

**CAR detection on electroporated T Cells.** Cells were washed and suspended in FACs buffer (PBS plus 0.1% sodium azide and 0.4% BSA). Biotin-labeled polyclonal goat anti-mouse F(ab)2 antibodies (anti-Fab, Jackson Immunoresearch, West Grove, PA) were added to the tube and the cells were incubated at 4°C for 25 minutes and washed twice. The cells were then stained with phycoerythrin-labeled streptavidin (BD Pharmingen, San Diego, CA).

**ELISA.** Target cells were washed and suspended at $10^6$ cells/mL in R10. One hundred thousand of each target cell type were added to each of 2 wells of a 96 well round bottom plate (Corning). Effector T cell cultures were washed and suspended at $10^6$ cells/mL in R10. One hundred thousand effector T cells were combined with target cells in the indicated wells of the 96 well plate. In addition, wells containing T cells alone were prepared. The plates were incubated at 37°C for 18 to 20 hours. After the incubation, supernatant was harvested and subjected to an ELISA assay using standard methods (Pierce, Rockford, IL).

**CD107a staining.** Cells were plated at an E:T of 1:1 ($10^5$ effectors:$10^5$ targets) in 160µl of complete RPMI medium in a 96 well plate. 20µl of phycoerythrin-labeled anti-CD107a Ab (BD Pharmingen, San Diego, CA) was added and the plate was incubated at 37°C for 1 hour before adding Golgi Stop and incubating for another 2.5 hours. After 2.5 hours 10 µl FITC-anti-CD8 and APC-anti-CD3 was added and incubated at 37°C for 30 min. After incubation, the samples were washed once with FACS buffer. Flow cytometry acquisition was performed with a BD FacsCalibur (BD Biosciences), and analysis was performed with FlowJo (Treestar Inc, Ashland, OR).

**Flow CTL.** A slightly modified version of a flow cytometry cytotoxicity assay was used (4). In this assay, the cytotoxicity of target cells is measured by comparing survival of target cells relative to the survival of negative control cells. The negative control cells and the target cells are combined in the same tube with effector T cells. Target cells were prepared by transducing parental K562 cells with human CD19 or mesothelin as described (1, 2). In our experiments, we tested the cytolytic effects of CD19-CAR T cells and Meso-CAR T cells using a mixture of both target cells (K562-CD19 or K562-meso). K562-meso were suspended in R10 medium at a concentration of $1.5 \times 10^6$ cells/mL, and the fluorescent dye CellTracker™ Orange CMRA (Invitrogen) was added at a concentration of 5 µM. The cells were mixed and then incubated at 37$^{O}$C for 30 minutes. The cells were then washed and suspended in R10. Next, the K562-meso were incubated at 37$^{o}$C for 60 min. The cells were then washed twice and suspended in R10. K562-CD19 were suspended in PBS+0.1% BSA at $1 \times 10^6$ cells/mL. The fluorescent dye carboxyfluorescein diacetate succinimidyl ester (CFSE) (Invitrogen) was added to this cell suspension at a concentration of 1 µM. The cells were incubated for 10 min at 37$^{o}$C. After the incubation, the labeling reaction was stopped by adding a volume of FBS that was equal to the

volume of cell suspension and the cells were incubated for 2 min at RT. The cells were washed and suspended in R10. Cultures were set up 96 well culture plate in duplicate at the following T cell:target cell ratios: 10:1, 3:1, and 1:1. The target cells were always 10,000 K562-meso in 0.1ml. Each culture also contained $10^4$ K562-CD19 negative control cells. In addition, cultures were set up that contained only K562-CD19 plus K562-meso cells. The cultures were incubated for 4 hrs at 37$^{o}$C. Immediately after the incubation, 7AAD (7-aminoactinomycin D) (BD Pharmingen) was added as recommended by the manufacturer, and flow cytometry acquisition was performed with a BD Calibur (BD Biosciences). Analysis was gated on 7AAD-negative (live) cells, and the percentages of live K562-CD19 and live K562-meso cells were determined for each T cell+target cell culture. The percent survival of K562-meso was determined by dividing the percent live K562-meso by the percent live K562-CD19 control cells. The corrected percent survival of K562-meso was calculated by dividing the percent survival of K562-meso in each T cell+target cell culture by the ratio of the percent K562-meso target cells:percent K562-meso negative control cells in tubes containing only K562-meso target cells and K562-CD19 control cells without any effector T cells. This correction was necessary to account for variation in the starting cell numbers and for spontaneous target cell death. Cytotoxicity was calculated as the percent cytotoxicity of K562-meso = 100 - corrected percent survival of K562-meso. For all effector:target ratios, the cytotoxicity was determined in duplicate and the results were averaged.

**Mouse xenograft studies.** Studies were performed as previously described with certain modifications (5, 6). Briefly, 6-10 week old NOD-SCID-$\gamma c^{-/-}$ (NSG) mice were obtained from the Jackson Laboratory (Bar Harbor, ME) or bred in house under an approved institutional animal care and use committee (IACUC) protocol and maintained under pathogen-free conditions. The derivation of M108 human mesothelioma from patient 108 was and used to

establish flank tumors using $5x10^6$ cells as previously described (1). M108 tumor cells were also engineered with a lentiviral vector to express firefly luciferase, yielding the M108-Luc cell line. Animals were injected intraperitoneally (IP) with $8x10^6$ viable M108-Luc. Tumor growth was monitored by size using caliper measurements for flank tumors, and by bioluminescent imaging and body weight for IP tumors.

**Bioluminescence imaging (BLI).** Tumor growth was monitored by BLI. Anesthetized mice were imaged using a Xenogen Spectrum system and Living Image v3.2 software. Mice were given an IP injection of 150 mg/kg body weight D-luciferin (Caliper Life Sciences, Hopkinton, MA) suspended in sterile PBS at a concentration of 15 mg/mL (100 µL luciferin solution/10 g mouse body weight). Previous titration of M108-Luc indicated the time to peak of photon emission to be five minutes, with peak emission lasting for 6-10 minutes. Each animal was imaged alone (for photon quantitation) or in groups of up to 5 mice (for display purposes) in the anterior-posterior prone position at the same relative time point after luciferin injection (6 minutes). Data were collected until the mid range of the linear scale was reached (600 to 60000 counts) or maximal exposure settings reached (f/stop 1, large binning and 1-2 seconds), and then converted to photons/second/$cm^2$/steradian to normalize each image for exposure time, f/stop, binning and animal size. For anatomic localization, a pseudocolor map representing light intensity was superimposed over the grayscale body-surface reference image. For data display purposes, mice without luciferase containing cells were imaged at maximal settings and a mean value of $3.6 x10^5$ p/s/$cm^2$/sr was obtained.

## Supplemental References

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