SUPPLEMENTARY MATERIALS AND METHODS

Primary cells and cell lines

Peripheral blood mononuclear cells (PBMC) were obtained from healthy donors by apheresis. Peripheral blood lymphocytes were isolated by centrifugal elutriation, and CD4+ and CD8+ T cells were purified by negative selection using the Rosette-Sep method for purification of CD4+ or CD8+ T cells (Stem Cell Technologies, Vancouver, BC) as described in the manufacturer's package insert. Cell purity routinely exceeded >95% by flow cytometry. Primary human T cells were cultured in RPMI 1640 media supplemented with 10% FCS, HEPES buffer (10 mM final concentration), penicillin (100 units/ml) and streptomycin (100 μg/ml). Primary pre-B acute lymphoblastic leukemia (ALL) cells (patient 240) were obtained from normally discarded cells obtained from individuals undergoing therapeutic apheresis for acute pre-B ALL. The isolated ALL cells were purified by Ficoll-Hypaque gradient centrifugation, and the cells were frozen in liquid N_2 in 10% DMSO until use. All primary cells were obtained under protocols approved by the Institutional Review Board of the University of Pennsylvania. Primary ALL cells were expanded for use by passage through immunodeficient mice.

The K562 cell line was used as an artificial antigen presenting cell for assessing CAR-mediated proliferation and cytokine production. K562 cells were obtained from the American Type Culture Collection. Cell lines expressing CD19 or CD32 (FcγRII) and 4-1BBL were generated by lentiviral vector-mediated transduction [1]. Lentiviral vectors expressing these proteins were generated by cloning of cDNA from PMBC through PCR and stand molecular biology techniques. All cDNA were confirmed by sequencing.

Expression of the receptors on the surface of the transduced K562 cells were periodically confirmed by flow cytometry, and found to be stable (data not shown).

Generation of high-titer lentiviral vectors and T cell transduction

The procedures for generation of high-titer lentiviral vectors have been previously described [2]. Briefly, 293T cells grown in RPMI with 10% FBS were co-transfected with lentiviral vector plasmids along with the pMDL.g/p, pRSV.rev and pVSVg packaging plasmids using Fugene (Roche, Indianapolis, IN) transfection reagent. Vector-containing supernatants were harvested at 24 and 48 hours after transfection, and concentrated by ultracentrifugation at 28,000 rpm for 2 hrs. Concentrated vector was stored at -70^{o}C until use. Vector titers were determined by a limiting dilution method on primary human CD4+ T cells activated with anti-CD3 and anti-CD28 coated beads. Titrations were performed in 96-well flat-bottom microtiter plates containing 100 μl of activated CD4+ T cells at 10^6 cells per mL. Vectors were diluted by 3-fold serial dilution, and 50 μl of diluted vector supernatant was added to different wells containing T cells. The percentage of T cells expressing the vector-encoded gene product was then determined by flow cytometry. The titer in transforming units per mL (TU/ml) for each vector was calculated from the vector dilution in which the percentage of positive cells was less than 20% but greater than 5% using the formula: $Titer = \%positive \times dilution \times 10^5 \times 20$

T cells were stimulated with anti-CD3 (clone OKT3) and anti-CD28 (clone 9.3) monoclonal antibodies immobilized on tosyl-activated paramagnetic beads (Invitrogen, Carlsbad, CA) at a cell to bead ratio of 3:1 as previously described. Following 24 hrs of activation, cells were transduced by addition of the high titer lentiviral vector at a multiplicity of infection of > 5 TU/cell for most experiments. Transduced T cells were

then maintained at a concentration of 0.8 x 10^6 cells/ml throughout the culture period by cell enumeration every 2-3 days. Cells were generally used in experiments when proliferation showed a decrease from log-phase and the mean lymphocyte volume decreased to 280-320 fL.

Assessment of cell proliferation and cytokine production

Assessment of CAR-mediated proliferation was performed in 12-well microtiter plates by mixing 10^6 washed T cells with $5x10^5$ K562 cells expressing CD19 (K19) or CD32 and CD137 (KT32-BBL) for a final T-cell:K562 ratio of 2:1. K562 cells were irradiated with 100 Gy of γ-radiation prior to use. Anti-CD3 (clone OKT3) and anti-CD28 (clone 9.3) monoclonal antibodies were added to cultures with KT32-BBL cells at 1 □g/mL to serve as a positive control for stimulating T-cell proliferation since these signals support long-term CD8+ T cell expansion ex vivo [3]. T cells were enumerated in cultures using CountBright™ fluorescent beads (Invitrogen, Carlsbad, CA) and flow cytometry as described by the manufacturer. CAR+ T cells were identified by GFP expression using T cells that were engineered with eGFP-2A linked CAR-expressing lentiviral vectors as described above. CD4+ and CD8+ expression on T cells were also simultaneously detected with specific monoclonal antibodies (BD Biosciences).

Cytokine measurements were performed on supernatants collected 24 hrs following re-stimulation using the human TH1/TH2 cytokine cytometric bead array kit (BD Biosciences, San Diego, CA) according the manufacturer's instructions. Fluorescence was assessed using a FACScalibur flow cytometer, and data was analyzed according to the manufacturer's instructions.

Cytotoxicity assays

Cytotoxicity was assessed by a standard ^{51}Cr-release assay. Target cells (K562 lines and primary pro-B-ALL cells) were loaded with 150 μCi ^{51}Cr (as $NaCrO_4$, New England Nuclear, Boston, MA) at 37^{o}C for 2 hrs with frequent agitation, washed twice in complete RPMI and plated into 96-well round-bottom microtiter plates at 5,000 cells per well in 100 μl. Effector T cells were mixed with target cells in the wells in complete RPMI at varying ratios of effector cell:target cell such that the total volume per well was 200 μl. Additional wells containing media only (spontaneous release, SR) or a 1% solution of triton-X 100 detergent (total release, TR) were also prepared. After 4 hrs of incubation at 37^{o}C, supernatant from each well was harvested. Released ^{51}Cr was then measured using a gamma particle counter (Packard Instrument Co., Waltham, MA). Each condition was performed in at least triplicate, and the percentage of lysis was calculated using the formula:

$$\% \; Lysis = \frac{E\,\mathrm{R} - SR}{TR - SR}$$

where ER represents the average ^{51}Cr released for each experimental condition.

Cell lysis, SDS-PAGE and Western Blot Analysis.

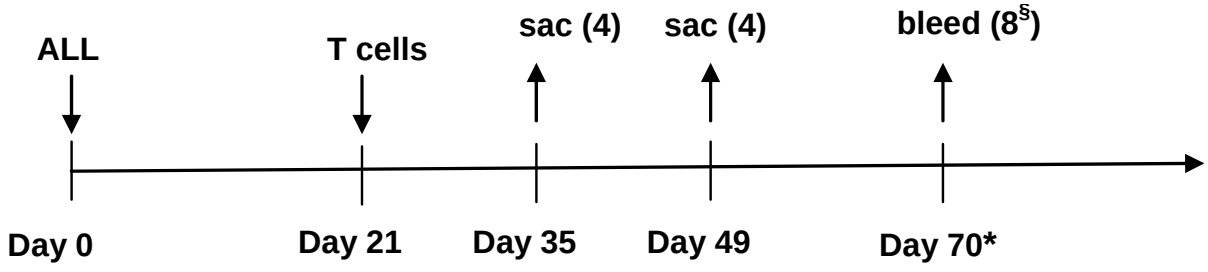
Cells were lysed in 1% NP-40 as described [2] and protease inhibitor cocktail (Sigma Chemical Co, St. Louis, MO) for 10 min at 4^{o}C. Following centrifugation at 10,000xg for 10 min, a reducing (containing 2-mercaptoethanol) or non-reducing loading buffer was added to the lysate. 3x10^{5} cell equivalents of lysate were separated on a 4-12% polyacrylamide gel using the NuPage PAGE system (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Following gel transfer to a nitrocellulose

membrane, the membrane was blocked with a solution containing 5% non-fat milk. CAR proteins were visualized using a monoclonal antibody to the TCR-ζ protein (clone 1ζ3A1, BD Biosciences, San Jose, CA), a goat-anti-mouse IgG secondary antibody conjugated to horseradish peroxidase (Sigma Chemical Co, St. Louis, MO) and chemiluminescence detection (ECL+ kit, Amersham) according to the manufacturer's instructions.

Competitive in vivo repopulation experiment with CAR T cells expressing αCD19-ζ and αCD19-BB-ζ.

This experiment was designed to evaluate the persistence and safety of CAR T cells in immune-deficient *NOD/scid* IL-2Rγc^{null} (NOG) mice. Male and female animals were distributed equally among the groups. Animals are purchased from Jackson Laboratories and were kept in microisolator cages under sterile conditions. All animal procedures are carried out with IACUC approval at the University of Pennsylvania.

NOG mice were engrafted with human acute B cell lymphocytic leukemia (B-ALL), followed two weeks later by escalating doses of a 1:1 mixture of CAR T cells expressing the αCD19-ζ and αCD19-BB-ζ receptors. Cohorts of the animals were sacrificed 3 and 5 weeks post T cell injection, and examined for the biodistribution of the CAR+ T cells via PCR for the vector. An additional cohort of animals was maintained indefinitely and screened periodically by flow cytometry on peripheral blood for the presence of peripheral blood T cells. The general experimental design is shown in Table S1 and the schematic below:



Primary B-ALL 240 leukemic cells were used as a tumor xenograft and to serve as a source of CD19 surrogate antigen were used as tumor antigen [4]. Tumor injection was done 2 weeks prior to T cell injection. Approximately 2 x 10^6 tumor cells were delivered via tail vein injection. In previous experiments, successful engraftment of B-ALL 240 cells into immunodeficient mice has been achieved at cell numbers as low as 1 x 10^6 viable cells per animal. These cells were obtained from the Stem Cell Leukemia Core of the University of Pennsylvania Cancer Center. Samples were collected under approval from the Institutional Review Board of the University of Pennsylvania after informed consent was given.

The T cells used for the competitive repopulation experiment were produced using lentiviral transduction and expansion ex vivo similar to our previous studies [5]. Briefly, T cells, obtained from a leukapheresis of a healthy donor under an IRB approved protocol were provided by the Human Immunology Core of the University of Pennsylvania. To prepare a 1:1 mixture of CAR T transduced human CD4 and CD8 T cells expressing TCR-ζ and TCR-ζ:4-1BB signaling domains, the T cells were depleted of monocytes and split into two cultures. Each T cell culture was stimulated with αCD3/αCD28coated beads and 24 hours post-stimulation, one culture was transduced with αCD19-TCR-ζ and the other with αCD19-TCR-ζ:4-1BB at an MOI of 1.9 or 2.8, respectively. On day 2, media was replaced with fresh media and cells were monitored on day 3, and then every other day for expansion and media replacement. Cultures were expanded to day 8, harvested, mixed at a 1:1 ratio, and injected into the mice at the doses indicated above. The harvested αCD19-TCR-ζ cells were 66.1% CD4+ and 32.9% CD8+; the harvested αCD19-TCR-ζ4-1BB cells were 70.3% CD4+ and 26.9% CD8+.

Expression of the chimeric immune receptor was measured by staining for the murine single chain variable fragment. The injected CAR T cells expressed the αCD19-TCR-ζ and αCD19-TCR-ζ:4-1BB constructs at 85.3% and 78.9%, respectively. Copy number was 4.6 αCD19-TCR-ζ copies and 3.4 αCD19-TCR-ζ:4-1BB copies per harvested cell. The cells were mixed 50:50, and the measured copy numbers were 2.4 TCR-ζ and 1.7 TCR-ζ:4-1BB copies relative to GAPDH in the injected mixture.

References for Supplemental Materials and Methods

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