Supplemental Methods

Anti-CD19-CAR-transduced T cell preparation

PBMC from each patient were suspended in AIM V medium (Invitrogen) with 5% human AB serum (Gemini) and 300 international units/mL IL-2 (Novartis). T cell proliferation was initiated by adding 50 ng/mL of the anti-CD3 monoclonal antibody OKT3 (Ortho Biotech). Six-well plates were coated with Retronectin™ (Takara Bio Inc.), and gammaretroviruses encoding the anti-CD19 CAR were coated onto the plates as previously described [1]. Two days after initiation of the PBMC cultures, $2x10^6$ stimulated PBMC were added to each well of the virus-coated plates, and the plates were cultured overnight. The next day, the transduction was repeated. Cells were infused into the patients on day 8 of culture. All cell products were assessed for potency by IFNγ release and for CAR expression by anti-Fab antibody staining. Cells were tested for sterility by bacteria culture, fungal culture, and mycoplasma PCR testing. Endotoxin was performed by limulus amoebocyte lysis assay, and replication competent retrovirus testing was performed by PCR. Cells were harvested and washed on day 8 of culture, and infused by bolus intravenous injection.

CAR detection on transduced T cells by anti-Fab antibody staining

This method was used in the experiments presented in Figure 1, and the percentages of CAR^+ $CD3^+$ cells listed in Table 2 were determined by this method. For each T cell

culture that was analyzed, one sample of cells was stained with biotin-labeled polyclonal goat anti-mouse-F(ab)$_2$ antibodies (anti-Fab, Jackson Immunoresearch) to detect the anti-CD19 CAR, and another sample of cells was stained with biotin-labeled normal polyclonal goat IgG antibodies (Jackson Immunoresearch) as an isotype control. The cells were then stained with phycoerythrin (PE)-labeled streptavidin (BD), anti-CD3 (eBioscience, clone UCHT1), anti-CD4 (eBioscience, clone OKT4), and anti-CD8 (eBioscience, clone RPA-T8). The percentage of T cells that expressed the CAR was calculated by subtracting the percentage of $CD3^+$ cells that were stained with the isotype control antibodies from the percentage of $CD3^+$ cells that were stained with the anti-Fab antibodies.

Target cell lines used

CD19-K562 are K562 cells (ATCC) transduced with full-length CD19 in our laboratory [2]. NGFR-K562 are K562 cells transduced with the gene for low-affinity nerve growth factor in our laboratory [2].

Enzyme-linked immunosorbent assay (ELISA)

For ELISAs to detect serum cytokines, patient serum from various time-points was assayed directly by using commercial ELISA kits (Pierce) according to the instructions of the manufacturer.

CD107a assay

Effector cells for CD107a assays were samples of the infused CAR-transduced T cells. For each effector cell sample that was tested, two tubes were prepared. One tube contained CD19-K562 cells, and the other tube contained NGFR-K562 cells. Both tubes contained effector cells, 1 ml of AIM-V medium+5% human serum, a titrated concentration of an anti-CD107a antibody (eBioscience, clone eBioH4A3), and 1 μL of Golgi Stop (monesin) (BD). All tubes were incubated at 37°C for 4 hours and then stained for CD3, CD4, and CD8.

Intracellular cytokine staining assay (ICCS)

Effector cells for ICCS assays were either samples of the infused CAR-transduced T cells or cryopreserved PBMC collected before treatment or after CAR-transduced-T-cell infusion. Prior to the assay, thawed PBMC were rested overnight in AIM-V medium+5% human serum without target cells and without cytokines at 37°C. For each type of effector cell that was tested, two tubes were prepared. One tube contained CD19-K562 cells and the other tube contained NGFR-K562 cells. Both tubes contained effector cells, 1 ml of AIM-V medium+5% human serum, and 1 μL of Golgi Plug (BD). All tubes were incubated at 37°C for 6 hours. The cells were surface stained with anti-CD3, anti-CD4, and anti-CD8. The cells were permeabilized, and intracellular staining was conducted for IFNγ (BD, clone B27), IL-2 (BD, clone MQ1-17H12), and TNF (BD, clone MAb11) by following the instructions of the Cytofix/Cytoperm kit (BD).

Ex vivo flow cytometry of B-lineage cells and T cell phenotype

Blood B cells were defined as $CD19^+$ cells. B-cell depletion was confirmed by staining for CD20. Blood T cells were defined as $CD3^+$ cells, and NK cells were defined as cells expressing CD16 and CD56 but not CD3. For T cell memory phenotype determination presented in Figure 1 and Supplemental Table 1, CAR^+ $CD3^+$ events were gated and the percentage of cells expressing memory markers was determined. Appropriate isotype control antibodies were used in all experiments. The memory antibodies used for the experiments reported in Figure 1 were anti-CD45RA (eBioscience, clone HI100) and anti-CCR7 (R&D Systems clone 150503).

For the T-cell phenotype data presented in Figure 5, freshly thawed PBMC were stained, and all plots are gated on live lymphocytes. The following antibodies were used in the experiment presented in Figure 5. An anti-CAR monoclonal antibody was provided by Laurence Cooper, M.D. Anderson Cancer Center.[3]

Manufacturer	Part number	Antigen and flourochrome
R&D	FAB197F	CCR7 FITC
Sigma	A-9400	7AAD
Ebioscience	25-0259-42	CD25 PE-Cy7
Ebioscience	17-1278-42	CD127 APC
BD	557943	CD3 Alexa700
Ebioscience	47-0458-42	CD45RA APC-e780
Ebioscience	48-0088-42	CD8 e450
Biolegend	317444	CD4 bv510
Biolegend	322306	CD57 FITC
Biolegend	329918	PD1 PE-Cy7
BD	559770	CD28 APC
Ebioscience	47-0279-42	CD27 APC e780

Bone marrow immunohistochemistry

Bone marrow biopsies were processed for morphologic evaluation by using standard procedures. The tissue was fixed in B-Plus fixative and decalcified. It was then paraffin-embedded. Immunohistochemical staining for CD79a, CD20 and CD19 was performed on biopsy sections with a Dako automated stainer, and the staining was visualized with Dako's Envision^{+} system. The clone LE-CD19 anti-CD19 antibody from Dako was used.

Real-time qPCR

For each patient, DNA was extracted from PBMC collected before treatment and at multiple time-points after treatment. DNA was extracted by using a Qiagen DNeasy blood and tissue kit. DNA from each time-point was amplified in duplicate with a primer and probe set (Applied Biosystems) that was specific for the anti-CD19 CAR. Real-time PCR was carried out with a Roche Light Cycler 480 real-time PCR system. Similar to an approach used previously by other investigators, we made serial 1:5 dilutions of DNA from the infused T cells of each patient into pretreatment DNA from the same patient, and we made standard curves by performing qPCR on this DNA [4, 5]. We determined the percentage of the infused T cells that expressed the anti-CD19 CAR by flow cytometry as detailed above under “CAR detection on transduced T cells by anti-Fab antibody staining”. We assumed that only infused T cells with surface CAR expression detected by flow cytometry contained the CAR gene. This assumption probably underestimates the actual number of cells containing the CAR gene because all cells

containing the CAR gene might not express the CAR protein on the cell surface. The percentage of PBMC that contained the CAR gene at each time-point was determined by comparing the qPCR results obtained with DNA of PBMC from each time-point to the qPCR results obtained from each patient's infused-T-cell standard curve. All samples were normalized to β-actin with an Applied Biosystems β-actin control reagents kit. After the percentage of CAR^+ PBMC was determined by PCR, the absolute number of CAR^+ PBMC was calculated by multiplying the percentage of CAR^+ PBMC by the sum of the absolute number of blood lymphocytes and monocytes.

References

1. Johnson LA, Morgan RA, Dudley ME, et al. Gene therapy with human and mouse T-cell receptors mediates cancer regression and targets normal tissues expressing cognate antigen. *Blood.* Jul 16 2009;114(3):535-546.

2. Kochenderfer JN, Feldman SA, Zhao Y, et al. Construction and preclinical evaluation of an anti-CD19 chimeric antigen receptor. *Journal of Immunotherapy.* Sep 2009;32(7):689-702.

3. Jena B, Maiti S, Huls H, et al. Chimeric Antigen Receptor (CAR)-Specific Monoclonal Antibody to Detect CD19-Specific T Cells in Clinical Trials. *PLoS ONE.* 2013;8(3).

4. Warren EH, Fujii N, Akatsuka Y, et al. Therapy of relapsed leukemia after allogeneic hematopoietic cell transplantation with T cells specific for minor histocompatibility antigens. *Blood.* 2010;115(19):3869-3878.

5. Morgan RA, Yang JC, Kitano M, Dudley ME, Laurencot CM, Rosenberg SA. Case report of a serious adverse event following the administration of t cells transduced with a chimeric antigen receptor recognizing ERBB2. *Molecular Therapy.* 2010;18(4):843-851.

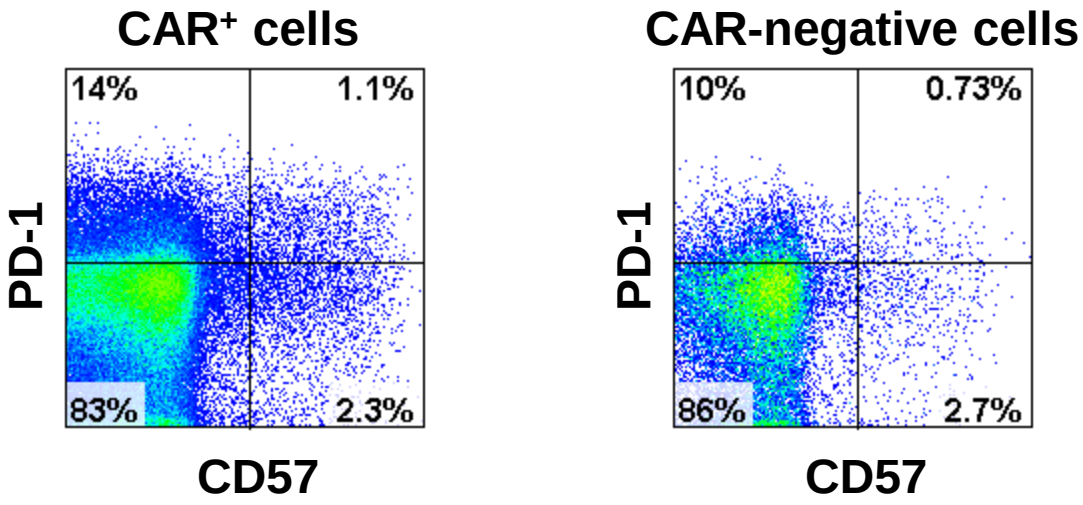
Supplemental Table 1

CD45RA and CCR7 phenotype of infused anti-CD19-CAR expressing T cells*

Patient	CD45RA+ CCR7+	CD45RA- CCR7+	CD45RA- CCR7-	CD45RA+ CCR7-
1	18.3	38.4	34.7	8.6
2	23.9	40.3	28.5	7.2
3	10.7	34.6	51.0	3.7
4	46.0	43.0	8.5	2.4
5	15.0	64.0	20.0	1.7
6	33.0	57.0	8.4	1.4
7	1.9	40.0	57.0	1.2
8	5.9	58.0	35.0	1.0
9	4.7	43.0	50.0	2.2
10	7.2	19	60	14

*A sample of the infused cells were stained with anti-CD3 and goat anti-mouse-Fab. CAR-expressing cells were stained with the goat anti-mouse-Fab antibody. The CD45RA and CCR7 phenotype of the CAR-expressing cells was determined.

Supplemental Figure 1



PD-1 and CD57 phenotype of the infused cells of Patient 1 are shown. The plots are gated on either CAR+ or CAR-negative cells. The plots are also gated on live $CD3^+$ lymphocytes.