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 stimulated 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 loaded 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 4 virus-coated plates, and the plates were cultured overnight. The next day, the transduction was repeated. Seven days later, cells that had undergone transduction were induced to proliferate by stimulating them with OKT3 in a rapid expansion protocol (REP) as previously described [1]. Cells were infused into the patients on day 14 after initiation of the REP.

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

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

NALM-6 (DSMZ) is a $CD19^+$ acute lymphoid leukemia line. 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].

Interferonγ ELISA

For ELISA assays to test cytokine production by CAR-transduced T cells, $CD19^+$ or CD19-negative target cells were combined with CAR-transduced T cells in duplicate wells of a 96 well round bottom plate (Corning) in AIM-V medium (Invitrogen)+5% human serum (Valley Biomedical). The plates were incubated at 37°C for 18-20 hours. Following the incubation, ELISAs for IFNγ, IL-2, and TNF were performed by using standard methods (Pierce, Rockford, IL).

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 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 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 were prepared as described under “CD107a assay”. 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 memory 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. The blood of Patient 3 was stained for CD19 and CD5 (Figure 3D) by using the manufacturer's recommended antibody concentrations. For T cell memory phenotype determination, 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 were, anti-CD45RA (eBioscience, clone HI100), anti-CCR7 (R&D Systems clone 150503), and anti-CD62L (BD, clone Dreg 56).

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 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 [3-4]. 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.

CD19 protein staining of CAR-tranduced T cells

PBMC samples from before treatment and after treatment were stained with titrated concentrations of DYKDDDDK-tagged human recombinant CD19 protein (Origene Technologies, Inc.) for 30 minutes at 4 °C. The cells were washed and then stained with a PE-labeled mouse anti-DYKDDDDK IgG antibody (Columbia Biosciences) plus anti-CD3, anti-CD4, and anti-CD8.

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. 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.

4. 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.

Table S1

Blood B-cell numbers 4 to 5 months after Anti-CD19-CAR–transduced T-cell infusion

	B cell/μL*
Patient 1aΨ	1
Patient 1bΨ	172
Patient 2	NE**
Patient 3	32
Patient 4	293
Patient 5	1245
Patient 6	1166
Patient 7	0
Patient 8	1

*B cells were defined as CD19+ cells (normal range 61-321/μL)

ΨPatient 1 was treated twice.

**Patient 2 was not evaluable due to death 18 days after CAR-transduced cell infusion.

Table S2

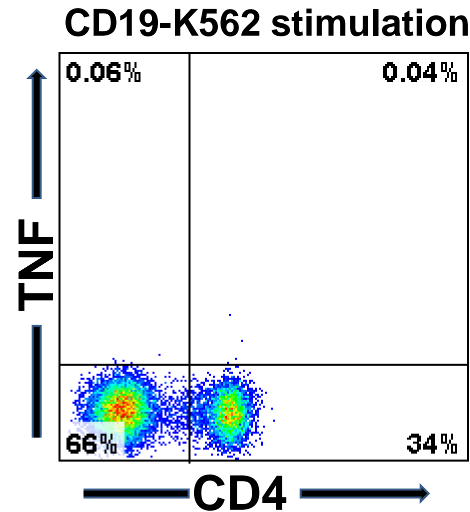
Body surface area of patients

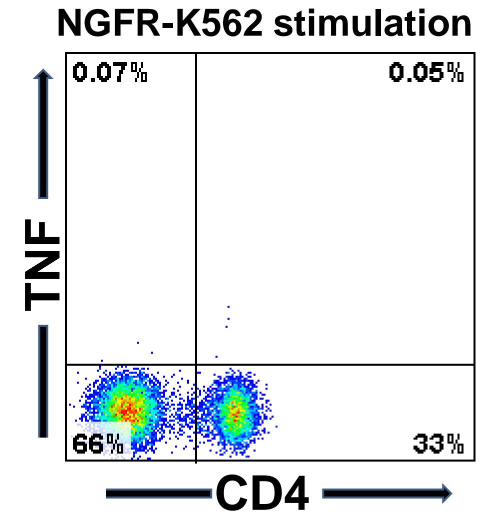
	Body surface area (m^2)
Patient 1aΨ	2.1
Patient 1bΨ	2.1
Patient 2	2.3
Patient 3	2.0
Patient 4	2.3
Patient 5	2.1
Patient 6	1.9
Patient 7	1.9
Patient 8	2.2

ΨPatient 1 was treated twice.

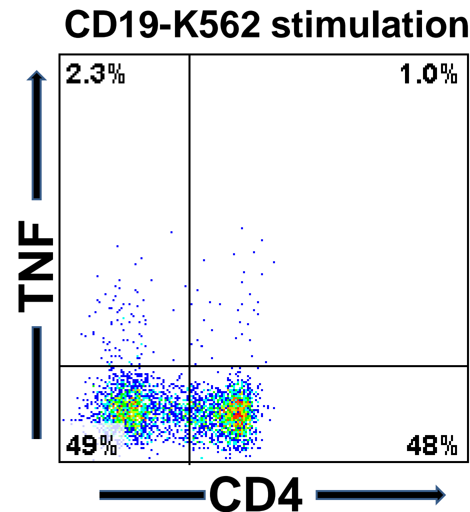
Figure S1

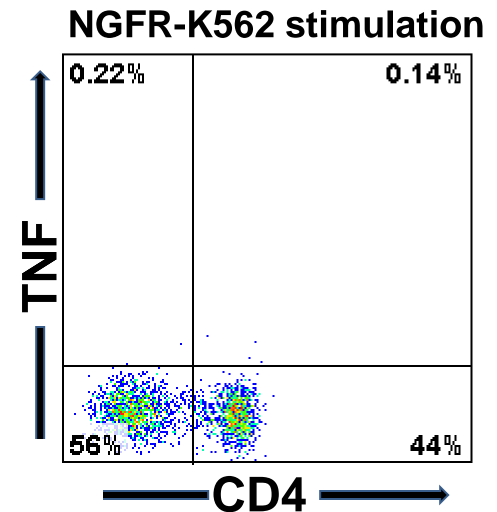
Pretreatment





13 days after infusion





PBMC were collected from Patient 8 thirteen days after CAR-transduced T-cell infusion. When the PBMC were cultured for 6 hours with CD19-K562 cells that express CD19, a population of T cells that produced TNF was detected; in contrast, when the PBMC were cultured with the negative control cells NGFR-K562 that lack CD19 expression, T cells did not produce TNF. PBMC collected before anti-CD19-CAR-transduced T-cell infusion did not produce TNF after a 6 hour incubation with either CD19-K562 or NGFR-K562. Plots are all gated on $CD3^+$ lymphocytes.