Anti-CD19-CAR-transduced T-cell preparation

The patient's PBMC 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. Otsu, Japan), and retroviruses 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 retrovirus-coated 6-well plates. The plates were centrifuged at 1000xg for 10 minutes and cultured overnight at 37°C. The next day, the transduction was repeated. After an overnight culture, the cells were removed from the plates and suspended in CM at $0.5x10^6$ cells/mL. Six days later, $12x10^6$ cells that had undergone transduction were induced to proliferate by stimulating them with OKT3 in a rapid expansion protocol (REP) as previously described[1] except autologous PBMC were used as feeder cells. The number of anti-CD19-CAR-transduced T cells increased by 336-fold during the REP, and cells were infused

into the patient on day 14 and day 15 after initiation of the REP. Prior to infusion, anti-CD19-CAR expression was detected by flow cytometry on 64% of the infused cells, which were 98% $CD3^{+}$ T cells (Supplementary Figure 1). CD19-specific interferonγ production by the transduced T cells was detected 3 days before the first infusion by enzyme-linked immunosorbent assay (ELISA) (Supplementary Table 1).

CAR detection on transduced T cells

Cells were washed and suspended in FACs buffer (phosphate-buffered saline (PBS) plus 0.1% sodium azide and 0.4% BSA). Fc receptors were blocked with normal goat IgG (Invitrogen). For each T cell culture to be analyzed, two tubes of cells were prepared. Biotin-labeled polyclonal goat anti-mouse-$F(ab)_2$ antibodies (anti-Fab, Jackson Immunoresearch) were added to one tube to detect the anti-CD19 CAR scFv, and biotin-labeled normal polyclonal goat IgG antibodies (Jackson Immunoresearch) were added to the other tube to serve as an isotype control. The cells were incubated at 4°C for 25 minutes and washed once. The cells were suspended in FACs buffer and blocked with normal mouse IgG (Invitrogen). The cells were then stained with phycoerythrin (PE)-labeled streptavidin (BD Pharmingen) along with anti-CD3 (eBiocience) and anti-CD8 (eBioscience). Flow cytometry acquisition was performed with a BD FacsCanto II (BD Biosciences), and analysis was performed with FlowJo (Treestar).

Target cell lines used

The following CD19-expressing immortalized cell lines were used: The acute lymphoid leukemia line NALM-6 (DSMZ), the B-cell diffuse large cell lymphoma line Toledo (ATCC), and CD19-K562. CD19-K562 were K562 cells (ATCC) transduced with full-length CD19 in our laboratory.[2] The CD19-negative T cell leukemia line CCRF-CEM (ATCC) and the CD19-negative line NGFR-K562, which are K562 cells transduced with the gene for low-affinity nerve growth factor in our laboratory[2], were used as negative control target cells.

Interferonγ enzyme-linked immunosorbent assay (ELISA)

Target cells were washed and suspended at 1 x10^6 cells per mL in T cell media without IL-2. One-hundred-thousand target cells of each target cell type were added to each of two wells of a 96 well round bottom plate (Corning). Effector T cell cultures were washed and suspended at 1 x10^6 cells per mL in T cell media without IL-2. 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 18-20 hours. Following the incubation, an IFNγ ELISA assay was performed using standard

methods (Pierce, Rockford, IL). Control effector cells used in this assay were transduced with a T-cell receptor targeting the melanoma antigen recognized by T cells-1 antigen (MART-1).

Ex vivo flow cytometry

B cells were stained for CD19 with the clone SJ25-4 antibody. T cells were defined as $CD3^+$ cells. NK cells were defined as cells expressing CD16 and CD56 but not CD3.

Specimens were stained with a panel of antibodies within 12 hours of collection. Erythrocytes were lysed by incubating with lysing solution (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) for 10 minutes at room temperature (maintained at 21-23 ^oC) at a ratio of 1:9 (volume of sample: volume of lysing solution). Specimens were then washed with phosphate buffered saline (PBS) to remove cytophilic antibodies before measuring viability by trypan blue uptake. Specimens were stained for 30 minutes at room temperature (maintained at 21-23 ^oC) using the manufacturer's recommended antibody concentrations according to Clinical Laboratory Standards Institute (CLSI) document H43-A recommendations.[3] After incubation, cells were pelleted by centrifugation (500xg for 15 minutes at room temperature), the media was aspirated, and the cells washed twice in a PBS solution containing 0.1% NaN_3 and 0.5% albumin. Cells were acquired after fixation in 1.0% paraformaldehyde and stored at 4^oC for up to

12 h before data acquisition. Normal lymphoid cells within specimens served as internal controls. Four-color flow cytometry was performed using a BD Biosciences FACSCalibur. The sensitivity of fluorescent detectors was monitored using CaliBRITE beads (BD Biosciences) according to the manufacturer's recommendations. Data (collected in list mode) were analyzed with CellQuest Pro software (BD Bioscience) and/or FCSExpress (De Novo Software). At least 5,000 lymphocytes were acquired per tube. Relevant cell populations were analyzed by gating on forward scatter, side scatter, and CD19.

Bone marrow histology and immunohistochemistry

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

Real-time PCR to quantitate CAR transgene

A primer and probe set that was specific for the variable regions of the anti-CD19 CAR was designed (Applied Biosystems). Quantitative real-time PCR was performed on genomic DNA with standard reagents and a Fast 7500 Real-time PCR System (Applied Biosystems). A standard curve was prepared for absolute quantitation of anti-CD19 CAR transgene copies by making serial dilutions of the plasmid that encoded the CAR in normal genomic DNA. A primer and probe set specific for β–actin was used to normalize for DNA quantity.

Figure S1. Transduced T cells expressed the anti-CD19 CAR

On day 11 of the rapid expansion protocol, cells that had been transduced with retroviruses encoding the anti-CD19 CAR and untransduced cells cultured in an identical manner were stained for CAR expression with goat anti-mouse Fab antibodies or with isotype-matched control antibodies. Cells were also stained with anti-CD3 and anti-CD8. The plots are gated on live $CD3^+$ lymphocytes, which made up 98% of the total cells. The numbers on the plots show the percentages of cells in each quadrant.

Figure S2. CD20$^+$ B cells are absent from the blood after anti-CD19 CAR-transduced T cell infusion. Peripheral blood CD20$^+$ cells were present before treatment, but no peripheral blood CD20$^+$ cells were detected by flow cytometry 28 weeks after anti-CD19-CAR-transduced T cell infusion. The numbers on the plots give the percentages of cells in each quadrant. The plots are gated on live lymphocytes.

Figure S3. Consistent with an eradication of B-lineage cells, a profound and prolonged decrease in serum immunoglobulin levels occurred after treatment. (A) The serum IgA levels are shown before treatment and at multiple times after treatment. After week 14, the patient started to receive infusions of intravenous immunoglobulins, which contain a small amount of IgA, so IgA levels after week 14 are not shown. Values of 10 mg/dL on the graph are below the limits of detection. (B) The serum IgM levels are shown before treatment and at multiple times after treatment. Values of 20 mg/dL on the graph are below the limits of detection.

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. 2009;114:535-546.
2. Kochenderfer JN, Feldman SA, Zhao Y, et al. Construction and preclinical evaluation of an anti-CD19 chimeric antigen receptor. J Immunother. 2009;32:689-702.
3. Stetler-Stevenson M, Ahmad E, Barnett D, et al. Clinical and Laboratory Standards Institute. Clinical Flow Cytometric Analysis of Neoplastic Hematolymphoid Cells;

Approved Guideline-Second Edition. Vol. H43-A2: Clinical and Laboratory Standards Institute; 2005.

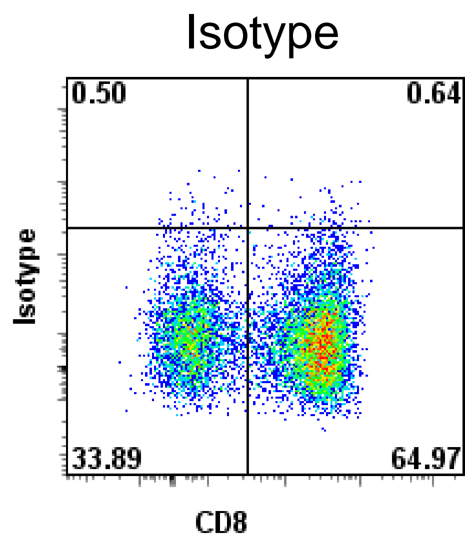
Table S1

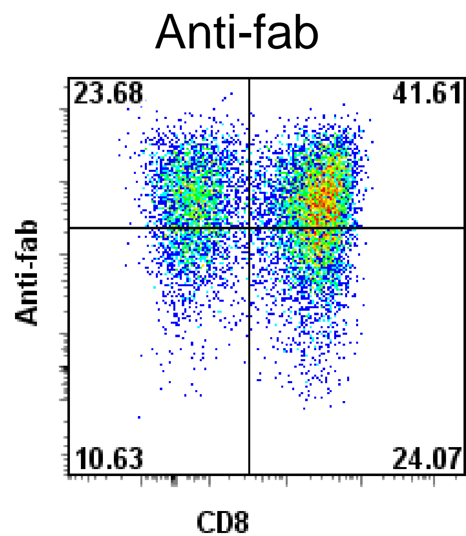
Effector cells*	CD19-expressing targets**			CD19-negative targets**		
	Toledo	Nalm6	CD19-K562	NGFR-K562	CCRL-CEM	Effectors alone
Patient 1 anti-CD19 CAR-transduced T cells	2180	4765	48050	581	193	110
Patient 1 Untransduced T cells	63	70	59	66	66	31
Control-transduced	0	434	5	3	19	3

*Assay was set up on day 11 of the rapid expansion protocol. **The values are the means of duplicate wells. The units are pg/mL of IFNγ.

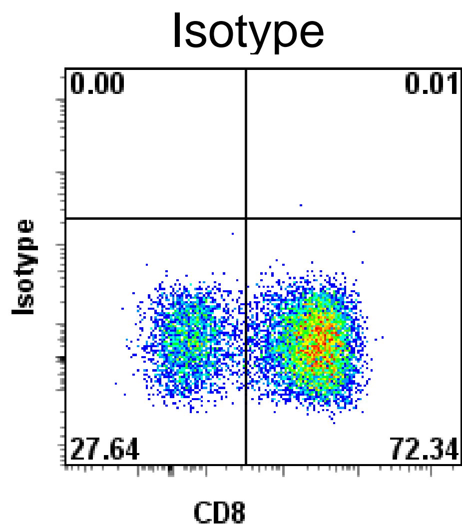
Figure S1

Anti-CD19-CAR transduced





Untransduced



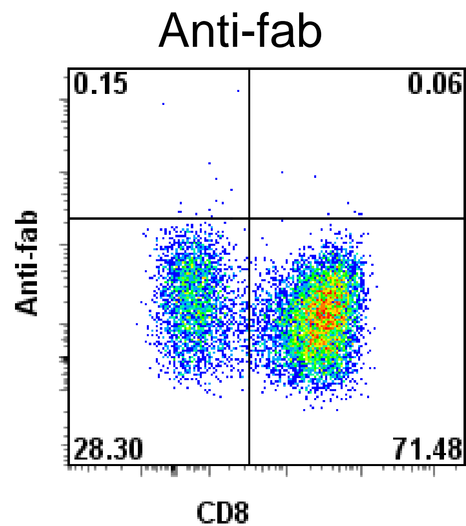
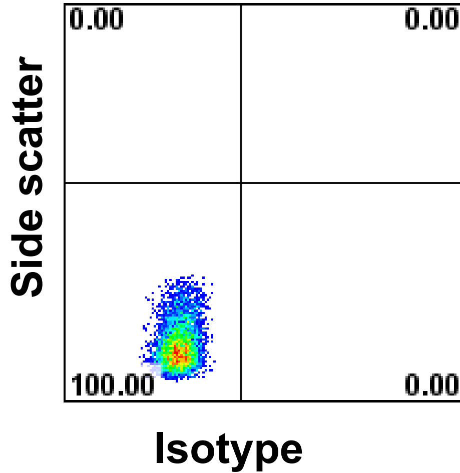
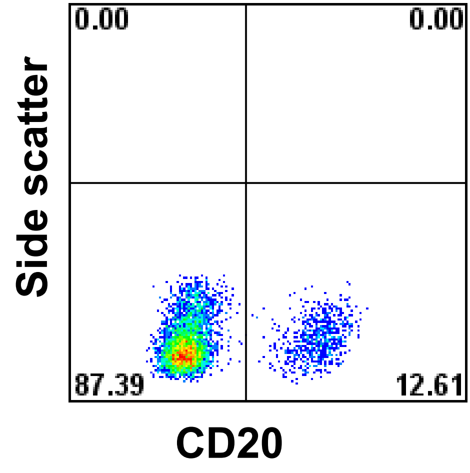


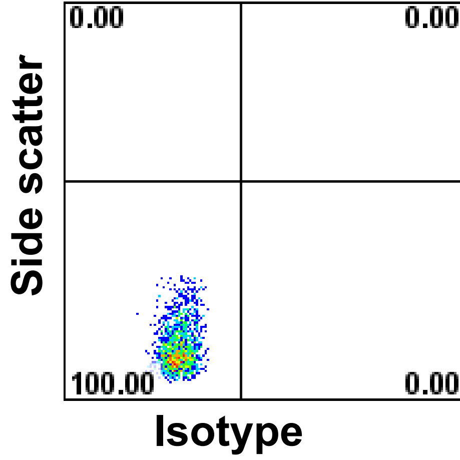
Figure S2

Pretreatment





28 weeks after treatment



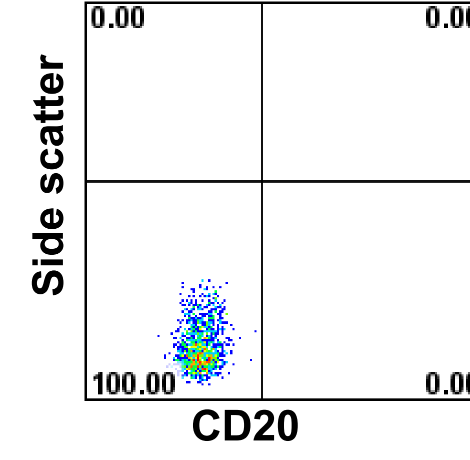


Figure S3

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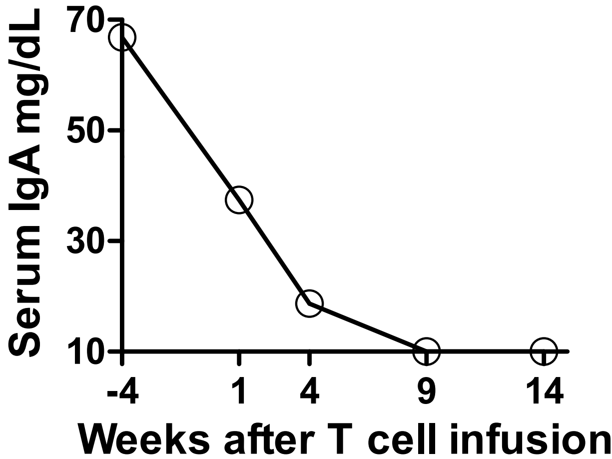

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