Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Maude SL, Frey N, Shaw PA, et al. Chimeric antigen receptor T cells for sustained remissions in leukemia. N Engl J Med 2014;371:1507-17. DOI: 10.1056/NEJMoa1407222

Sustained Remissions with Chimeric Antigen Receptor T Cells For Leukemia

Shannon L. Maude, M.D., Ph.D.,* Noelle Frey, M.D., M.S.C.E.,* Pamela A. Shaw, Ph.D., Richard Aplenc, M.D., Ph.D., David M. Barrett, M.D., Ph.D., Nancy J. Bunin, M.D., Anne Chew, Ph.D., Vanessa E. Gonzalez, M.B.A., Zhaohui Zheng, M.S., Simon F. Lacey, Ph.D., Yolanda D. Mahnke, Ph.D., Jan J. Melenhorst, Ph.D., Susan R. Rheingold, M.D., Angela Shen, M.D., David T. Teachey, M.D., Bruce L. Levine, Ph.D., Carl H. June, M.D., David L. Porter, M.D., and Stephan A. Grupp, M.D., Ph.D.

Division of Oncology, The Children's Hospital of Philadelphia, PA (S.L.M., R.A., D.M.B, N.J.B., S.R.R., D.T.T., S.A.G.)
Department of Pediatrics (S.L.M., R.A., D.M.B, N.J.B., S.R.R., D.T.T., S.A.G.), Division of Hematology-Oncology (N.F., D.L.P.), Departments of Pathology and Laboratory Medicine (J.J.M., B.L.L., C.H.J., S.A.G.) and Biostatistics and Epidemiology (P.A.S., R.A.), and Abramson Cancer Center (N.F., A.C., V.E.G., Z.Z., S.F.L., Y.D.M., J.J.M., B.L.L., C.H.J., D.L.P., S.A.G.), University of Pennsylvania Perelman School of Medicine, all in Philadelphia, PA
Novartis Pharmaceutical Corporation, East Hanover, NJ (A.S.)

Supplemental Appendix

Contents

Supplemental Methods

Trial Design

Pilot clinical trials designed to assess the safety and feasibility of CTL019 T cell therapy in relapsed/refractory CD19+ malignancies were conducted at the Children's Hospital of Philadelphia (ClinicalTrials.gov number, NCT01626495) and the University of Pennsylvania (ClinicalTrials.gov number, NCT01029366). Written informed consent for participation was obtained from patients or their guardians according to the Declaration of Helsinki and protocols were approved by the institutional review boards of the Children's Hospital of Philadelphia and the Hospital of the University of Pennsylvania, respectively.

Patients with relapsed or refractory CD19+ malignancies who were ineligible for allogeneic stem cell transplant (allo SCT) or who relapsed after a prior allo SCT were eligible to enroll. Eligibility criteria included adequate kidney and liver function and a successful test expansion of an aliquot of peripheral blood mononuclear cells in response to CD3/CD28 stimulation. Patients with prior allo SCT were eligible, provided 6 months had elapsed from SCT, they had no evidence of graft-versus-host disease and did not require immunosuppression at the time of enrollment or infusion. Patients with active CNS involvement (CNS3), uncontrolled active infection, active hepatitis B or C, or HIV infection were excluded.

Study Procedures

Patients underwent leukapheresis at the time of relapse or after one or more reinduction attempts. Patients with prior allo SCT were eligible, and T cells were collected from the patient, not the donor. Median donor chimerism in the T cell compartment was 100%. One week prior to infusion, patients received a chemotherapy regimen aimed at lymphodepletion (Table S1). Patients were infused with 1-10x10^7 T cells/kg (5-50x10^8 T cells for patients ≥ 50 kg) over 1-3 days. Response was assessed by bone marrow (all patients) and CSF evaluation (pediatric trial only) at 1, 3, 6, 9, and 12 months, after which disease surveillance was limited to analysis of peripheral blood unless there were clinical concerns. Complete remission (CR) was defined by morphologic assessment of the bone marrow as M1 (<5% leukemic blasts) with no evidence of extramedullary disease. Minimal residual disease (MRD) was assessed by multiparametric flow cytometry at the University of Washington. An interim analysis of 30 patients treated from April 2012 to February 2014 was conducted with a data cutoff of April 18, 2014.

General laboratory statement

CTL019 T cells were produced in the Clinical Cell and Vaccine Production Facility and research sample processing, freezing, and laboratory analyses were performed in the Translational and Correlative Studies Laboratory, both at the University of Pennsylvania. Both laboratories operate under principles of Good Manufacturing Practices and Good Clinical Laboratory Practice with established Standard Operating

Procedures (SOPs) and/or protocols for sample receipt, processing, freezing, and analysis. Assay performance and data reporting conforms with MIATA guidelines.[1]

Production of CTL019 T cells

Peripheral blood mononuclear cells (PBMCs) were collected by leukapheresis, T cells were enriched by mononuclear cell elutriation, washed, and expanded by addition of anti-CD3/CD28–coated paramagnetic beads for activation of T cells.[2] The lentiviral vector containing the previously described CD19-BB-ζ transgene[2] (produced by the Vector Core at the Children's Hospital of Philadelphia) was added at the time of cell activation and was washed out on day 3 after culture initiation.[3] Cells were expanded on a rocking platform device (WAVE Bioreactor System) for 8 to 12 days. On the final day of culture, the beads were removed by passage over a magnetic field and the CTL019 cells were harvested and cryopreserved in infusible medium. Final product release criteria in the IND included the following: cell viability ≥ 70%, CD3+ cells ≥ 80%, residual paramagnetic anti-CD3/CD28-coated paramagnetic beads ≤ 100 per $3x10^6$ cells, Endotoxin ≤ 3.5 EU/mL, Mycoplasma negative, Bacterial and fungal cultures negative, residual bovine serum albumin ≤ 1 μg/mL, VSV-G DNA as a surrogate marker for replication competent lentivirus ≤ 50 copies per μg DNA, transduction efficiency by flow cytometry ≥ 2%, transduction efficiency by vector DNA sequence 0.02 to 4 copies per cell.

Sample processing

Samples (peripheral blood, bone marrow) were collected in lavender top (K2EDTA) or red top (no additive) vacutainer tubes (Becton Dickinson). Lavender top tubes were delivered to the laboratory within 2 hours of the sample draw, or shipped overnight at room temperature in insulated containers as described.[4] Samples were processed within 16 hours of drawing according to the established SOP. Peripheral blood (PBMC) and bone marrow mononuclear cells (BMMC) were purified, processed, and stored in liquid nitrogen as described.[5] Red top tubes were processed within 2 hours of the draw including coagulation time; serum was isolated by centrifugation, aliquoted in single use 130 μL aliquots and stored at -80°C. Cerebrospinal fluid (CSF) was delivered to the laboratory within 30 minutes of collection and cells were pelleted by centrifugation and processed for quantitative PCR and flow cytometry.

Soluble factor analysis

Quantification of soluble factors including cytokines was performed using Luminex bead array technology (Life Technologies). Assays were performed as per the manufacturer's protocol with a 9-point standard curve generated using a 3-fold dilution series. The 2 external standard points were evaluated in duplicate and the 5 internal standards in singlicate; all samples were evaluated in duplicate at 1:2 dilution; calculated %CVs for the duplicate measures were less than 15%. Data were acquired on a FlexMAP-3D instrument (Luminex Corp.) and analyzed using XPonent 4.0 software and 5-parameter logistic regression analysis. Standard curve quantification ranges were determined by

the 80-120% (observed/expected value) range. Reported values included those within the standard curve range and those calculated by the logistic regression analysis.

Flow cytometry

Cells were evaluated by flow cytometry directly after Ficoll-Paque processing, with the exception of the Patient 2 baseline sample, which was evaluated immediately after thawing of a cryopreserved sample. Immunophenotyping of peripheral blood and marrow samples was performed using approximately 2-5x10^5 total cells per condition (depending on cell yield in samples), and for CSF samples using any cells collected following centrifugation of CSF fluid. Fluorescence minus one (FMO) controls were used for CTL019 evaluation. Antibodies for T cell detection panels: anti-$CD3^{FITC}$ (clone UCHT1), anti-$CD8^{PE}$ (clone RPA-T8), anti-$CD14^{PE\text{-}Cy7}$ (clone 61D3), anti-$CD16^{PE\text{-}Cy7}$ (clone CB16), and anti-$CD19^{PE\text{-}Cy7}$ (clone HIB19) (all from eBioscience), as well as MDA-$CAR4^{Alexa647}$, a murine antibody specific for the idiotype of CTL019 (a generous gift of Drs. Bipulendu Jena and Laurence Cooper, MD Anderson Cancer Center).[6] Antibodies for B cell detection panels: anti-$CD20^{FITC}$ (clone 2H7), anti-$CD45^{PE}$ (clone 2D1), anti-$CD10^{PE\text{-}Cy7}$ (clone CB-CALLA), anti-$CD19^{APC}$ (clone HIB19), and anti-$CD34^{PE\text{-}Cy7}$ (clone 4H11) (all from eBioscience). Cells were incubated in 163 μL PBS containing 1% fetal bovine serum and 0.02% NaN_3 as well as the Abs for 30 minutes on ice, washed, resuspended in PBS containing 0.5% paraformaldehyde, and acquired using an Accuri C6 cytometer equipped with a blue (488 nm) and red (633 nm) laser. Data were analyzed using either BD Accuri C6 Analysis (BD Biosciences) or FlowJo software (Version 10, Treestar). Compensation values were established using BD compensation

beads (BD Biosciences). The gating strategy for T cells was as follows: Intact cells (FSC/SSC) ➔ dump channel (CD14+CD16+CD19-PECy7) vs. CD3+ ➔ CD3+ (Fig. S1A). The general gating strategy for B cells was as follows: Intact cells (FSC/SSC) ➔ SSC low events ➔ CD19+ (Fig. S1B).

Quantitative (q) PCR analysis

Genomic DNA was isolated directly from whole blood and qPCR analysis performed using ABI Taqman technology and a validated assay to detect the integrated CD19 CAR transgene sequence as described[1] using 200 ng genomic DNA per time-point for peripheral blood and marrow samples. To determine copy number per unit DNA, an 8-point standard curve was generated consisting of 5 to 10^6 copies CTL019 lentivirus plasmid spiked into 100 ng non-transduced control genomic DNA. The number of copies of plasmid present in the standard curve was verified using digital qPCR with the same CD19 CAR primer/probe set, and performed on a QuantStudio™ 3D digital PCR instrument (Life Technologies). Each data-point (sample, standard curve) was evaluated in triplicate with a positive Ct value in 3/3 replicates with % CV less than 0.95% for all quantifiable values. To control for the quality of interrogated DNA, a parallel amplification reaction was performed using 20 ng genomic DNA, and a primer/probe combination specific for a non-transcribed genomic sequence upstream of the CDKN1A (p21) gene as described.[5] These amplification reactions generated a correction factor to adjust for calculated versus actual DNA input. Copies of transgene per microgram DNA were calculated according to the formula: Copies/microgram

genomic DNA = copies calculated from CTL019 standard curve x correction factor/amount DNA evaluated (ng) x 1000 ng.

Statistical Analyses

For time-to-event analyses, Kaplan-Meier curves were used to estimate survival distributions; log-rank tests were used to detect between-group differences in survival curves. Peak levels of inflammatory markers in the 28-day interval following first infusion were calculated. Wilcoxon rank sum test was used to detect associations between biomarkers and CRS severity, including baseline disease burden and 28-day peaks for inflammatory markers. Levels below the limit of detection were imputed as half the lower limit of detection. Statistical analyses were performed using R (Version 3.0.1. Vienna: R Development Core Team). All statistical tests were two-sided and performed at the 0.05 significance level.

Author roles

In addition to study conduct (SLM, NF, RA, DMB, NJB, AC, SRR, DTT, CHJ, DLP, SAG), SAG, DLP and CHJ designed the study, SLM, NF, PAS, RA, DMB, NJB, AC, VEG, ZZ, SFL, BLL, YDM, JJM, SRR, DTT gathered the data, SAG, SLM, PAS, CHJ, DLP, NF, RA, DMB, NJB, AS, SFL, BLL, YDM, JJM analyzed the data, PAS, SLM, SAG and CHJ vouch for the data and the analysis, SLM, CHJ and SAG wrote the paper, and SAG, DLP, AS and CHJ decided to publish the paper. No non-author wrote the first draft or any part of the paper.

Supplemental Results

Inflammatory Markers of Cytokine Release Syndrome

We observed a constellation of symptoms, physical findings, and laboratory abnormalities in patients experiencing cytokine release syndrome that was similar to patients with macrophage activation syndrome (MAS) or hemophagocytic lymphohistiocytosis (HLH),[7,8] disorders of excessive immune activation.[9] Hyperferritinemia is a hallmark of HLH/MAS; therefore, we prospectively monitored ferritin levels in patients treated with CTL019 and found marked elevations in ferritin during the period of maximal T cell proliferation.

Patients with severe cytokine release syndrome had higher peak levels of CRP ($P=0.022$), ferritin ($P=0.005$), IL-6 ($P<0.001$), IFNγ ($P<0.001$), and IL-2R ($P<0.001$), compared to patients without severe CRS (Figures 4 and S2). While Davila *et al*. recently reported that CRP ≥20 mg/dL was associated with severe CRS in patients treated with 19-28z CAR T cells,[10] in our cohort, only 50% of patients with CRP ≥20 mg/dL had severe CRS; therefore, its utility in CRS management may depend on CAR design.

CD19 Antigen Density

In order to assess whether clinical outcome was related to CD19 expression levels on the patient's leukemia cells, we measured the anti-CD19 antibody-binding capacity (ABC) by flow cytometry. Keeping in mind that only 3/30 patients were non-responders, we did not find a difference in anti-CD19 ABC between CR and NR: in CR it ranged

from 8,032 to 60,448 (median: 23,327), while in NR it was 9,611, 16,928 and 57,039. CD19 negative cells (T cells) had a background ABC of 781.

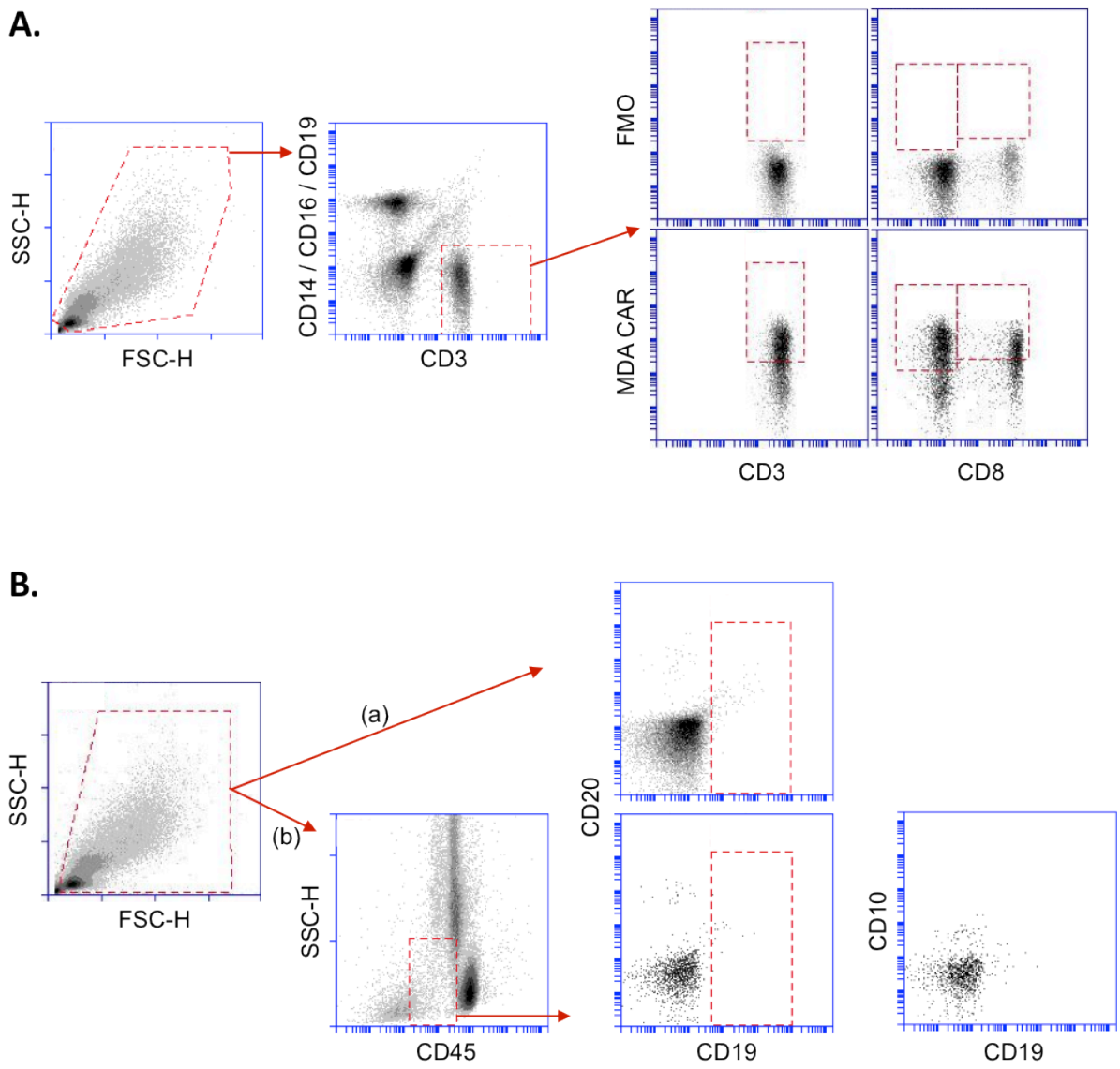

Figure S1. Gating schemes. A. Intact cells are selected in a FSC-H vs. SSC-H dot plot. T cells are identified as $CD3^{+}$ $CD14^{-}$ $CD16^{-}$ $CD19^{-}$ and investigated for CAR expression with a monoclonal antibody which recognizes the CAR epitope (MDA CAR mAb); gates are set on an FMO control (upper plots). **B.** Intact cells (FSC-H vs. SSC-H) are investigated for total $CD19^{+}$ cells to assess B-cell aplasia (a). Additionally, $CD45^{dim}$ cells are interrogated for the presence of ALL cells (b). The example dot plots are from a PBMC sample taken 15 days after CTL019 infusion.

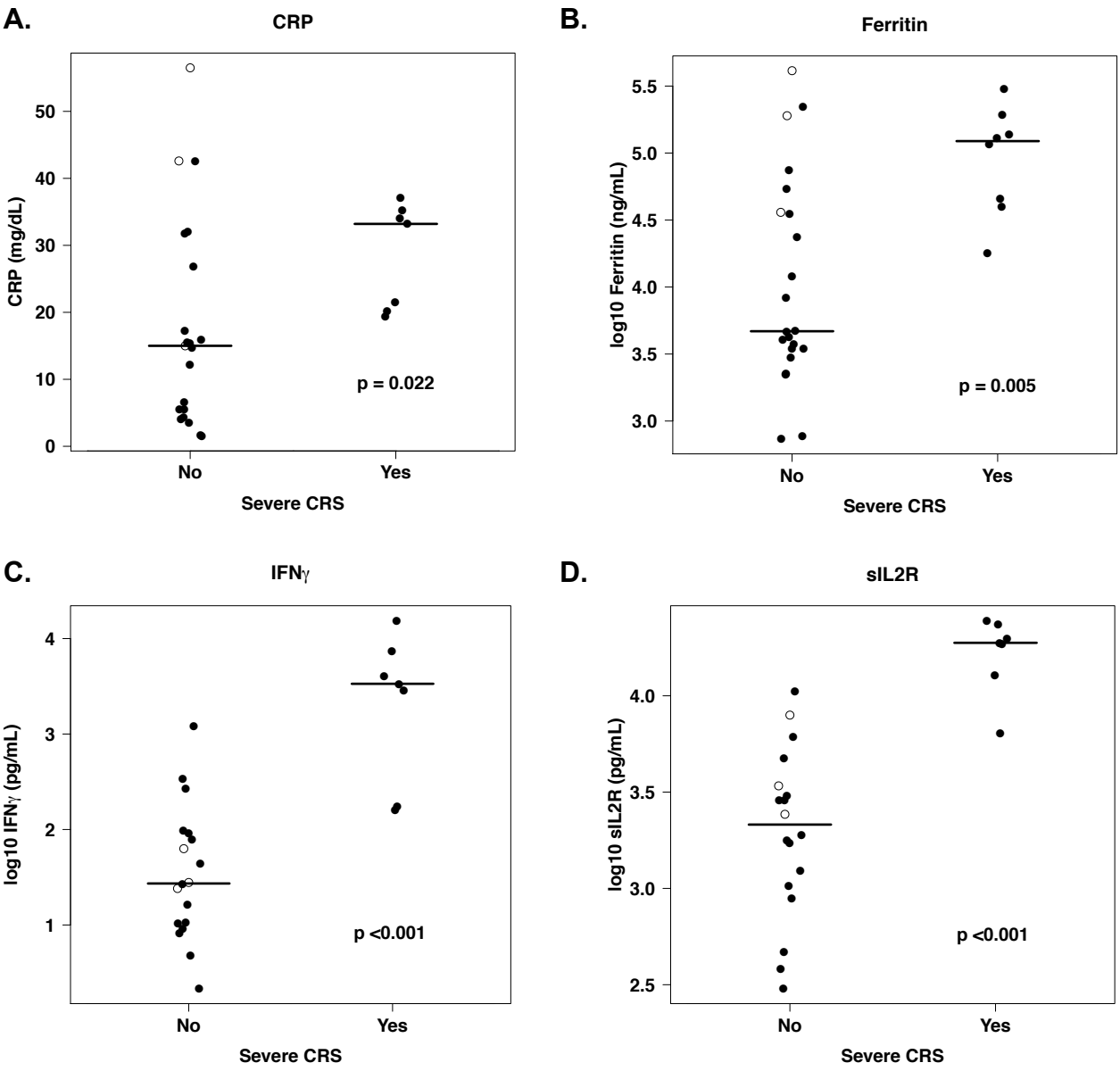

Figure S2. Inflammatory markers and cytokines in CRS. Peak levels of CRP **(A)**, ferritin **(B)**, IFNγ **(C)**, and sIL2R **(D)** in first 28 days for patients with severe CRS compared to patients with non-severe CRS. Severe CRS was defined as hypotension requiring 2 or more vasopressors or respiratory failure requiring mechanical ventilation. Closed circle: complete remission (CR); open circle: no response (NR); line: median.

Table S1. Lymphodepleting chemotherapy regimens

Subject ID	Chemotherapy Dose and Schedule
1	None
2	Cy/VP[1]
3	Cy/VP
4	Etoposide 150 mg/m^2 daily x 1 day, Cytarabine 300 mg/m^2 daily x 1 day
5	None
6	Flu/Cy[2]
7	Cy/VP
8	Cy/VP
9	None
10	Flu/Cy-B[3]
11	Flu/Cy-B
12	Cy/VP
13	Flu/Cy
14	Cyclophosphamide 1000 mg/m^2 daily x 1 day
15	Flu/Cy
16	Flu/Cy
17	Flu/Cy
18	Flu/Cy
19	Flu/Cy
20	Flu/Cy
21	Flu/Cy
22	Flu/Cy
23	Flu/Cy
24	Flu/Cy
25	Flu/Cy
26	Clofarabine 30 mg/m^2 daily x 5 days
27	CVAD-B[4]
28	CVAD-A[5]
29	Cyclophosphamide 300 mg/m^2 every 12 hours x 3 days
30	Cyclophosphamide 300 mg/m^2 every 12 hours x 3 days

Chemotherapy aimed at lymphodepletion was given 1 week prior to CTL019 infusion, timed so the last dose was given 2-6 days prior to infusion. The chemotherapy regimen was individualized based on prior patient response to chemotherapy. Three patients with pancytopenia did not receive lymphodepleting chemotherapy.

[1]Cy/VP: Etoposide 100 mg/m^2 daily x 2 days, Cyclophosphamide 440 mg/m^2 daily x 2 days

[2]Flu/Cy: Fludarabine 30 mg/m^2 daily x 4 days, Cyclophosphamide 500 mg/m^2 daily x 2 days

[3]Flu/Cy-B: Fludarabine 30 mg/m^2 daily x 3 days, Cyclophosphamide 300 mg/m^2 daily x 3 days

[4]CVAD-B: Methotrexate 1000 mg/m^2 day 1, Cytarabine 1000 mg/m^2 every 12 hours days 2, 3

[5]CVAD-A: Cyclophosphamide 300 mg/m^2 every 12 hours days 1-3, Vincristine 2 mg day 3, Adriamycin 50 mg/m^2 day 3

Table S2. CTL019 manufacture and dose

Subject ID	PBMC Input ($x10^8$)	PBMC Yield ($x10^{10}$)	Percent transduced	CTL019+ cells infused Total ($x10^8$)	CTL019+ cells infused $x10^6$/kg	Response	Severe CRS (Y/N)
1	4.50	0.73	11.6%	3.78	11.55	CR	Y
2	3.66	1.16	14.4%	0.39	1.52	CR	N
3	4.00	1.48	18.3%	0.38	1.86	NR	N
4	4.30	1.43	25.3%	0.48	2.54	CR	N
5	0.91	1.06	35.9%	0.86	3.59	CR	N
6	3.61	1.67	21.4%	4.28	5.94	CR	N
7	2.00	1.75	16.2%	0.30	1.62	NR	N
8	4.50	1.57	10.7%	0.34	1.07	CR	N
9	0.30	0.18	42.7%	1.45	4.27	CR	Y
10	3.60	2.74	45.3%	9.06	17.36	CR	N
11	4.50	1.29	30.4%	1.26	3.04	CR	Y
12	4.40	2.58	35.3%	1.73	3.53	CR	Y
13	4.50	2.41	20.9%	3.68	8.35	CR	N
14	4.50	0.93	21.5%	3.58	8.61	NR	N
15	3.70	1.89	37.4%	3.83	14.96	CR	N
16	3.50	0.75	38.8%	7.76	6.63	CR	Y
17[1]	4.50	2.36	32.6%	1.63	2.74	CR	N
18	2.14	1.46	34.0%	4.53	13.60	CR	N
19	3.88	0.30	22.8%	2.06	9.12	CR	N
20	4.50	0.79	33.8%	6.76	11.99	CR	Y
21	2.67	0.38	18.3%	1.43	7.32	CR	N
22	4.50	1.20	19.5%	3.43	7.80	CR	N
23	4.50	0.05	33.8%	1.01	1.58	CR	N
24	4.50	1.93	16.4%	0.54	1.64	CR	Y
25	4.50	3.18	10.3%	2.06	2.48	CR	N
26	4.50	1.47	13.5%	0.68	0.79	CR	Y
27	4.18	1.44	14.6%	0.73	0.76	CR	N
28	3.50	0.41	31.1%	9.58	12.24	CR	N
29	1.28	1.54	5.5%	2.76	3.62	CR	N
30	2.20	1.31	21.4%	1.07	1.80	CR	N

PBMC, peripheral blood mononuclear cells; CR, complete remission; NR, no response

[1]Subsequent infusions at 3 and 6 months for total dose of $2.06x10^7$ CTL019 cells/kg

Supplemental References

1. Janetzki S, Britten CM, Kalos M, et al. "MIATA"-minimal information about T cell assays. Immunity 2009;31:527-8.

2. Milone MC, Fish JD, Carpenito C, et al. Chimeric receptors containing CD137 signal transduction domains mediate enhanced survival of T cells and increased antileukemic efficacy in vivo. Mol Ther 2009;17:1453-64.

3. Levine BL, Humeau LM, Boyer J, et al. Gene transfer in humans using a conditionally replicating lentiviral vector. Proceedings of the National Academy of Sciences 2006;103:17372-7.

4. Olson WC, Smolkin ME, Farris EM, et al. Shipping blood to a central laboratory in multicenter clinical trials: effect of ambient temperature on specimen temperature, and effects of temperature on mononuclear cell yield, viability and immunologic function. J Transl Med 2011;9:26.

5. Kalos M, Levine BL, Porter DL, et al. T Cells with Chimeric Antigen Receptors Have Potent Antitumor Effects and Can Establish Memory in Patients with Advanced Leukemia. Science Translational Medicine 2011;3:95ra73.

6. 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:e57838.

7. Grupp SA, Kalos M, Barrett D, et al. Chimeric Antigen Receptor–Modified T Cells for Acute Lymphoid Leukemia. New England Journal of Medicine 2013;368:1509-18.

8. Teachey DT, Rheingold SR, Maude SL, et al. Cytokine release syndrome after blinatumomab treatment related to abnormal macrophage activation and ameliorated with cytokine-directed therapy. Blood 2013;121:5154-7.

9. Risma K, Jordan MB. Hemophagocytic lymphohistiocytosis: updates and evolving concepts. Curr Opin Pediatr 2012;24:9-15.

10. Davila ML, Riviere I, Wang X, et al. Efficacy and Toxicity Management of 19-28z CAR T Cell Therapy in B Cell Acute Lymphoblastic Leukemia. Science Translational Medicine 2014;6:224ra25.