Allogeneic T-cells expressing an anti-CD19 chimeric antigen receptor induce remissions of B-cell malignancies that progress after allogeneic hematopoietic stem cell transplantation without causing graft-versus-host disease

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Supplemental Methods

CAR19 T cell preparation

PBMC from each patient were suspended in AIM V medium (Life Technologies) with 5% human AB serum (Valley Biomedical) and 300 international units (IU)/mL IL-2 (Prometheus). T cell proliferation was initiated by adding 50 ng/mL of the anti-CD3 monoclonal antibody OKT3 (Miltenyi). Six-well plates were coated with Retronectin™ (Takara Bio Inc.), and gammaretroviruses encoding CAR19 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. Cells were removed from the plates and allowed to proliferate in vitro in AIM V medium with 5% human serum and 300 IU/mL IL-2. Cells were infused into the patients on day 8 of culture. All cell products were assessed for potency by interferon gamma (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 testing was performed by limulus amoebocyte lysate assay (Lonza), and replication competent retrovirus testing was performed by PCR.

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

This method was used to determine the percentage of CAR^+ T cells in the infused T cells. The percentages of CAR^+ $CD3^+$ cells listed in Table 2 were determined by this

method. For each patient, a sample of donor-derived CAR19-transduced cells was stained with biotin-labeled polyclonal goat anti-mouse-F(ab)$_2$ antibodies (anti-Fab, Jackson Immunoresearch) to detect the CAR, and a sample of untransduced identically-cultured cells from the same donor was stained with the anti-Fab antibodies as a control. Next, the cells were all 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 CAR19-transduced T cells that expressed the CAR was calculated by subtracting the percentage of untransduced $CD3^+$ cells that were stained with the anti-Fab antibodies from the percentage of CAR19-transduced $CD3^+$ cells that were stained with the anti-Fab antibodies.

In vitro recognition of CLL cells by donor CAR19 T cells

PBMC from Patient 11's unrelated donor were either transduced and cultured as described above under "CAR19 T cell preparation" above or cultured in an identical manner without being transduced with the gene for CAR19. Both types of cells were cultured for 8 days. Next, the CD19 CAR-transduced and the untransduced cells were separately combined with PBMC collected from Patient 11 at a time before CAR19 T-cells were infused. These PBMC were mostly CLL cells. The combined T-cell+CLL cell cultures were continued for 9 days in AIM V medium with 5% human serum and 150 IU of IL-2. After 9 days of culture, the T cells were co-cultured overnight with either Patient 11 PBMC from before the CAR T-cell infusion or Patient 11 PBMC from 11 days after the CAR19 T-cell infusion. The PBMC from before the CAR T-cell infusion were mostly

CLL while the PBMC from 11 days after the infusion contained no CLL cells or normal B cells. After the overnight co-culture, a standard IFNγ ELISA was performed.

Enzyme-linked immunosorbent assay (ELISA) for IL-6

IL-6 ELISAs were performed on serum by using commercial ELISA kits (Life Tech) according to the instructions of the manufacturer.

Ex vivo flow cytometry

Blood B cells were defined as $CD19^+$ cells. Blood T cells were defined as $CD3^+$ cells, and NK cells were defined as cells expressing CD56 but not CD3. For the T-cell phenotype data presented in Figure 4, freshly thawed PBMC were stained, and all plots are gated on live lymphocytes. The following antibodies were used in the experiment presented in Figure 4. For some antigens 2 different antibodies are listed, but in any one experiment the same antibody was used for all samples. An anti-CAR monoclonal antibody was provided by Laurence Cooper, M.D. Anderson Cancer Center.[2]

Manufacturer	Part number	Antigen and flourochrome
R&D	FAB197F	CCR7 FITC or APC
Sigma	A-9400	7AAD
BD	557943	CD3 Alexa700
Ebioscience	47-0458-42	CD45RA APC-e780
BD	555488	CD45RA FITC
Ebioscience	48-0088-42	CD8 e450
BD	563256	CD8 bv510
Biolegend	317444	CD4 bv510
Biolegend	300518	CD4 APC-Cy7
Biolegend	329918	PD1 PE-Cy7

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 was performed on biopsy sections with a Ventana Benchmark Ultra automated stainer according to the manufacturer's instructions. Wright-Giemsa and terminal deoxynucleotidyl transferase (TdT) staining were performed by using standard methods.

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 CAR19. 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 expressing CAR19 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. To determine the percentage of PBMC that contained the CAR gene at each time-point, we compared the qPCR results obtained with DNA of PBMC from each time-point to the qPCR results obtained from each patient's infused 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.* 2009;114(3):535-546.
2. 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).
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.

Supplemental Table 1: Dose escalation for allogeneic anti-CD19 CAR T cells.

Dose Level	Number of anti-CD19 CAR T cells infused for HLA-identical and one-antigen mismatched sibling stem cell transplant cohort (cells/kg recipient body weight)	Number of anti-CD19 CAR T cells infused for matched unrelated donor stem cell transplant cohort (cells/kg recipient body weight)
Dose Level 1	$1x10^6$ - $5x10^6$	$0.5x10^6$ - $1.5x10^6$
Dose Level 2	$5.1x10^6$ - $1x10^7$	$1.6x10^6$ - $5x10^6$
Dose Level 3	NA	$1x10^7$

Separate dose escalations were conducted for recipients of URD transplants and recipients of sibling transplants. Each dose level included a range of possible cell doses with the final dose being determined by the principle investigator for each patient.

Supplemental Table 2: Patients enrolled in the study who did not receive anti-CD19 CAR T-cell infusion

Patient number	Reason CAR T-cell therapy not administered
Untreated 1	Performance status deterioration due to progressive malignancy
Untreated 2	Performance status deterioration due to progressive malignancy
Untreated 3	The patient voluntarily withdrew consent.
Untreated 4	Donor cryopreserved cells previously available from an outside institution were found to be depleted, and the donor became medically ineligible for another collection.
Untreated 5	A patient with a history of paroxysmal atrial flutter developed atrial flutter with rapid ventricular response. The patient was not treated due to concerns of cardiac toxicity in this setting.
Untreated 6	The patient acutely developed a transaminitis and other signs of progressive graft versus host disease, and was no longer eligible to participate.

Supplemental Table 3: Post-transplant therapies received by patients before CAR19 T-cell protocol enrollment

Patient	Malignancy	Prior Post-Transplant Treatments	Number of Post-transplant therapies including chemotherapy, immunotherapy, DLIs, and courses of radiation	Number of Prior DLIs	T-cell dose of last standard DLI before enrollment on CAR19 protocol (CD3+ cells/kg)	Time from last standard DLI before CAR19 T-cell infusion in months	Response to last DLI before enrollment on CAR19 protocol
1	CLL	R-EPOCH Pentostatin-cyclophosphamide-rituximab Radiation therapy BEAM-dexamethasone-Alemtuzumab Tumor derived lymphocyte infusion, experimental therapy[1] Rituximab* DLI	11	5	$5.9x10^7$	5	SD
2	DLBCL	R-EPOCH* DLI	2	1	$2.5x10^7$	3	SD
3	CLL	Pentostatin-cyclophosphamide-rituximab x 5 EPOCH-FR (vincristine omitted) x 2 R-EPOCH (vincristine omitted) Bendamustine-rituximab x 2* DLI	24	14	$2.9x10^7$	8	PD
4	DLBCL	IVAC* Radiation therapy DLI	3	1	$1.1x10^7$	2	SD
5	CLL	Fludarabine-cyclophosphamide-ofatumumab Cyclophosphamide -	8	5	$1.0\ x10^8$	13	SD

		doxorubicin- vincristine Hyper-CVAD* DLI Note: The patient had 2 prior HLA-matched sibling allogeneic transplants before her URD transplant. Post-transplant relapse therapies listed here refer to those received after her most recent (URD) transplant.					
6	MCL	Rituximab x 3 lenalidomide Rituximab-Lenalidomide* DLI	8	3	$5.0x10^7$	15	PR
7	CLL	DLI only Note: The patient had one prior HLA-matched sibling allogeneic transplant before his URD transplant. The post-transplant DLI is his only post URD transplant therapy.	1	1	$1.0x10^7$	5	PD
8	MCL	Rituximab x 2 Lenalidomide R-EPOCH Bendamustine-rituximab-Bortezomib* DLI	6	1	$3.6x10^7$	12	PR
9	MCL	EPOCH-FR Bendamustine-rituximab Rituximab Radiation therapy x 2 Dexamethasone-lenalidomide-rituximab Ofatumumab* DLI	9	2	$1.0x10^6$	9	SD
10	MCL	Radiation therapy Pentostatin-cyclophosphamide-rituximab	9	6	$3.1x10^7$	25	SD

		R-EPOCH* DLI					
11	CLL	Ofatumumab* DLI	5	4	$5x10^7$	4	PD
12	ALL Ph+	Imatinib Clofarabine* DLI	3	1	$1x10^7$	4	Failed to obtain CR
13	MCL	Rituximab-cytarabine - bortezomib Ibrutinib Rituximab-bendamustine-cytarabine* DLI	4	1	$1x10^7$	14	PD
14	ALL Ph-neg	Rituximab-fludarabine-cyclophosphamide-etoposide Cyclophosphamide+Autologous anti-CD19 CAR T cells Ibrutinib*	3	0	NA	NA	NA
15	ALL Ph-neg	Vincristine -prednisone-L-asparaginase-mitoxantrone-bortezomib Vincristine-prednisone-L-aspariginase Asparaginase – low-dose methotrexate* Note: The patient underwent two myeloablative HLA-matched sibling allogeneic transplants from the same donor. Listed therapies follow the more recent transplant.	3	0	NA	NA	NA
16	ALL Ph-neg	Hyper-CVAD Vincristine Hyper-CVAD Part B (methotrexate and cytarabine) Fludarabine-cyclophosphamide -etoposide*	4	0	NA	NA	NA
17	DLBCL	Ibrutinib*	3	2	$2.5x10^7$	5	PR

		DLI**[2]					
18	DLBCL	DLIs only**[2]	1	1	$1x10^7$	3	PD
19	FL transformed to DLBCL	Rituximab x 2 Radiation therapy x 3 Bendamustine Rituximab-fludarabine-cyclophosphamide Dexamethasone R-ICE* DLI	11	2	$2.5x10^7$	12	SD
20	ALL Ph-	Vincristine* Experimental Donor derived NK cells x 2[3]	3	0	NA	NA	NA

CLL --chronic lymphocytic leukemia. DLBCL -- diffuse large B-cell lymphoma. MCL -- mantle cell lymphoma. ALL Ph+ -- Philadelphia chromosome positive acute lymphoblastic leukemia. ALL Ph-neg -- Philadelphia chromosome negative acute lymphoblastic leukemia. FL -- follicular lymphoma. URD -- unrelated donor allogeneic stem cell transplant. *Most recent chemo-immunotherapy. **All prior donor lymphocyte infusions were composed of donor $CD4^+$ T cells cultured ex vivo using CD3/CD28 co-stimulation and IL-4, IL-2, and rapamycin; received as an experimental therapy on a clinical trial protocol.[2] DLI -- donor lymphocyte infusion. x 2 or x 3 -- Indicates that patient received two or three courses of the chemotherapy regimen at different times with restaging assessment following each course. BEAM -- carmustine, etoposide, cytarabine, melphalan. IVAC -- ifosfamide, cytarabine, etoposide, intrathecal methotrexate. HyperCVAD -- hyperfractionated cyclophosphamide, vincristine, doxorubicin, dexamethasone. R-EPOCH -- rituximab, etoposide, prednisone, vincristine, cyclophosphamide, doxorubicin. EPOCH-FR etoposide, prednisone, vincristine, doxorubicin, cyclophosphamide, fludarabine, rituximab. R-ICE -- rituximab, ifosfamide, carboplatin, etoposide. SD -- stable disease. PD -- progressive disease. PR -- partial remission. NA -- not applicable.

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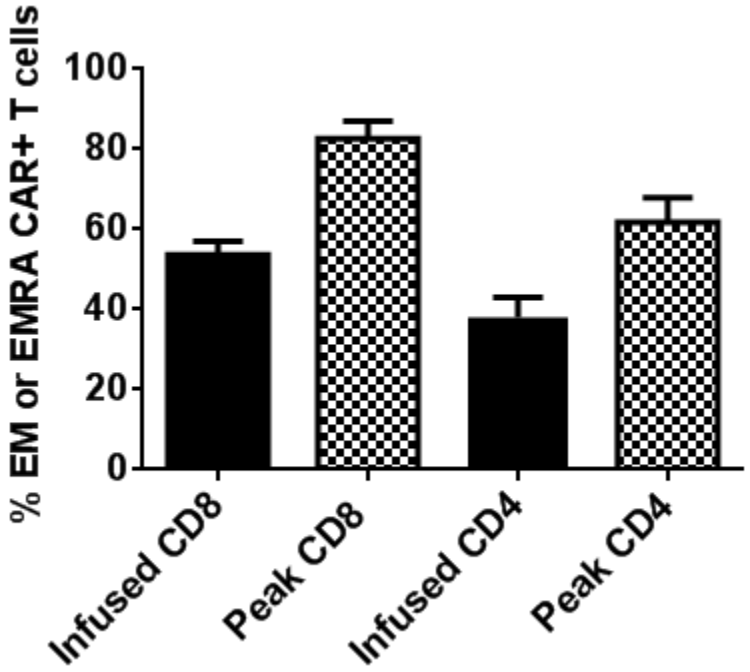
Supplemental Table 4: Donor chimerism prior to CAR19 T-cell infusion

Patient Number	Recipient blood $CD3^+$ cell chimerism^ at the time of CAR T-cell infusion (% of $CD3^+$cells of donor origin)
1	93
2	100
3	100
4	100
5	100
6	100
7	100
8	100
9	100
10	94
11	99
12	100
13	100
14	100
15	100
16	81*
17	100
18	100
19	100
20	100

^Chimerism determined by standard PCR methods.

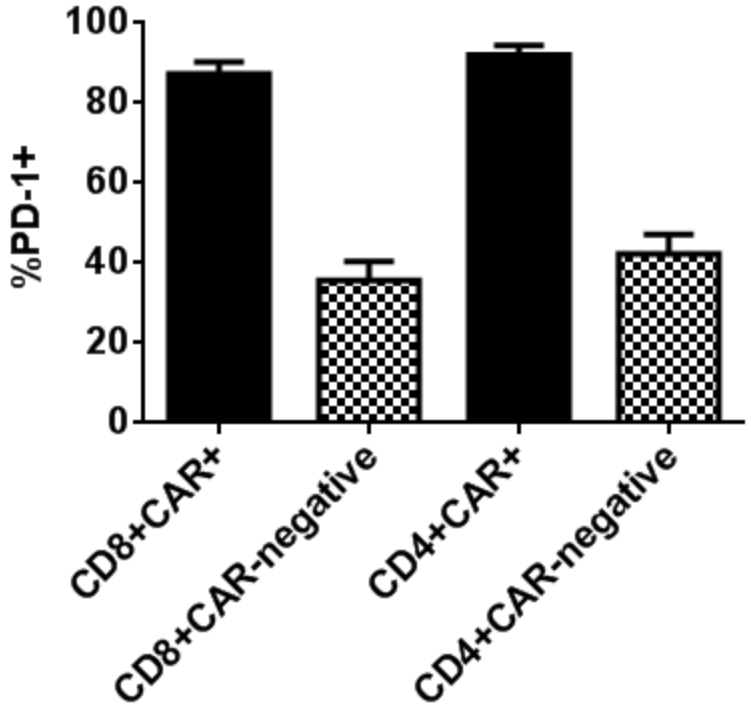
*Bone marrow total white blood cell chimerism.

Supplemental Figure 1



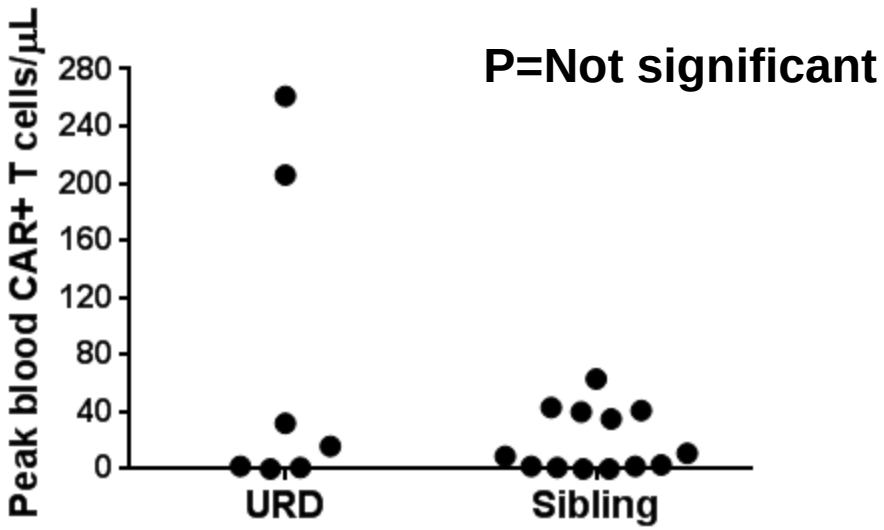
Flow cytometry was performed on a sample of the infused T cells (Infused) or on PBMC from the time of peak CAR19 levels between 5 and 14 days after infusion (Peak). Effector Memory (EM) T cells were defined as cells with a CD45RA-negative, CCR7–negative phenotype. Effector memory RA T cells were defined as cells with a CD45RA^{+}, CCR7-negative phenotype. The mean plus standard error of the mean (SEM) are shown for each category. The Wilcoxon matched-pairs signed rank test was used to compare the infused vs peak blood EM or EMRA CAR T-cell levels ($P<0.0001$ for the CD8 infused versus peak comparison and $P<0.0001$ for the CD4 infused versus peak comparison). The analysis was performed on all 16 patients with detectable blood CAR19 T cells after infusion and available blood samples.

Supplemental Figure 2



PBMC from the time of peak blood CAR19 levels, 5-14 days after infusion, were stained with an anti-CAR antibody and antibodies against CD3, CD4, CD8, and PD-1. The fractions of $CD3^+CAR^+CD8^+$, $CD3^+CAR^+CD4^+$, $CD3^+$CAR-negative$CD8^+$, and $CD3^+$CAR-negative$CD4^+$ cells that expressed PD-1 are shown. For both $CD8^+$ T cells and $CD4^+$ T cells, the fraction of cells expressing PD-1 was higher for CAR-expressing T cells than for CAR-negative T cells.

Supplemental Table 3



A statistically-significant difference in peak blood CAR19 T cell levels was not found between recipients of unrelated donor transplants (URD) and recipients of HLA-matched sibling transplants (Sibling). CAR19 T cells were quantitated by qPCR. The groups were compared with the Mann Whitney test.