Chemotherapy-refractory Diffuse Large B-cell Lymphoma and Indolent B-cell Malignancies Can Be Effectively Treated with Autologous T Cells Expressing an Anti-CD19 Chimeric Antigen Receptor

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Supplemental Methods

Anti-CD19-CAR 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 cells were returned to culture in AIM V medium with 5% human AB serum and 300 international units/mL of IL-2. Cells were infused into the patients on day 10 of culture. All cell products were assessed for potency by IFNγ release and for CAR expression by anti-Fab antibody staining. Release criteria for clinical T-cell products were at least 200 pg/mL of IFNγ release against $CD19^+$ targets in a standard enzyme-linked immunosorbant assay (ELISA) and at least 30% CAR expression on T cells as measured by anti-Fab flow cytometry. Cells were tested for sterility by bacteria culture, fungal culture, and mycoplasma PCR testing. Endotoxin testing was performed by limulus amoebocyte lysis assay, and replication competent retrovirus testing was performed by PCR. Cells were harvested and washed on day 10 of culture, and infused by intravenous infusion over 20 to 30 minutes.

CAR detection on transduced T cell cultures by anti-Fab antibody staining

This method was used to determine the percentage of CAR^+ cells, and this percentage was used in calculating the total number of T cells to infuse into patients to achieve the target CAR^+ T cell doses. An example of this staining is shown in Figure 1. For each T cell culture that was analyzed, cells were stained with biotin-labeled polyclonal goat anti-mouse-$F(ab)_2$ antibodies (anti-Fab, Jackson Immunoresearch) to detect the anti-CD19 CAR. The cells were then stained with phycoerythrin (PE)-labeled streptavidin (BD), anti-CD3, anti-CD4, and anti-CD8. The percentage of T cells that expressed the CAR was calculated by subtracting the percentage of untransduced $CD3^+$ cells that were stained with the anti-Fab antibody from the percentage of anti-CD19 CAR-transduced $CD3^+$ cells that were stained with the anti-Fab antibodies. Both the anti-CD19-CAR-transduced T cells and the untransduced T cells came from the same patient and were cultured identically.

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 samples of the infused CAR-transduced T cells. 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. Blood T cells were defined as $CD3^+$ cells. For the T-cell memory phenotype determination presented in Figure 5, live lymphocytes that were CAR^+ and $CD3^+$ were gated, and the percentage of cells expressing the various markers was determined on $CD4^+$ and $CD8^+$ cells. For the T-cell phenotype data presented in Figure 5, freshly thawed PBMC were stained with an anti-CAR monoclonal antibody that was provided by B. Jena and L. Cooper, M.D. Anderson Cancer Center.[3] This antibody was used to stain the lymph node mass aspirate shown in Figure 3 and to stain patient PBMC samples shown in Figure 5. The absolute number of blood CAR^+CD3^+ cells was calculated by multiplying the peripheral blood lymphocyte count by the percentage of live lymphocytes that were $CD3^+CAR^+$ as determined in the flow cytometry analysis. The following antibodies were used in the experiment presented in Figure 5.

Manufacturer	Part number	Antigen and flourochrome
eBioscience	11-0458-71	CD45RA FITC
R&D	FAB197A	CCR7 APC
BD	557943	CD3 Alexa700
Ebioscience	48-0088-42	CD8 e450
Biolegend	317444	CD4 bv510
Biolegend	322306	CD57 FITC
Biolegend	329918	PD1 PE-Cy7

Real-time qPCR to quantitate blood CAR$^+$ cells

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 and as published previously in our own work, 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 [4-7]. 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". The standard curve was in units of %CAR+ T cells. The highest point of the standard curve was the point derived from undiluted infused T-cell DNA. 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.

Brain qPCR

To determine the CD19 cDNA copy number, a quantified DNA plasmid that contained the sequence of full-length CD19 was serially diluted with salmon sperm DNA solution (10ng/µl) to use in generating a standard curve. The standard curve DNA and the human brain array cDNA were amplified by using TaqMan Fast Universal PCR Master Mix (2x) (Part Number. 4352042, Applied Biosystems). The following 20x premade primers/probe mix was used for CD19: Hs01047413-g1(Invitrogen)**.** Results were normalized to the expression of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping gene. GAPDH qPCR was performed with this primer and probe set: GAPDH-HMTM-Forward (TCGGAGTCAACGGATTTGG), 8 µM; GAPDH-HMTM-Reverse (CAATATCCACTTTACCAGAGTTAAAAGC), 8 µM; GAPDH-HMTM-probe (FAM-TTGGGCGCCTGGTCACCAGGG-Blackhole), 5 µM. The following human brain cDNA was used: TissueScan Human Brain Tissue qPCR Array (HBRT101, OriGene Technologies, Inc.)

Brain Immunohistochemistry

A normal brain tissue array (US Biomax part number BNC17011) was stained with the clone LE-CD19 anti-CD19 antibody from Dako.

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.

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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.

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6. Kochenderfer JN, Dudley ME, Feldman SA, et al. B-cell depletion and remissions of malignancy along with cytokine-associated toxicity in a clinical trial of anti-CD19 chimeric-antigen-receptor-transduced T cells. *Blood.* 2012;119(12):2709-2720.

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Supplemental Table 1

Patient lymphocyte counts at the time of apheresis

Patient	Blood lymphocyte count at the time of apheresis (cells/μL)*
1	560
2	140
3	460
4	200
5	630
6	180
7	1470
8	280
9	550
10	1270
11	320
12	340
13	1350
14	640
15	1020

*Normal range of blood lymphocytes is 1320-3570/μL.

Supplemental Table 2

Phenotype of Infused Cells

Patient	% CD3+ cells expressing the CAR**	% of infused cells that were CD8+^	% of infused cells that were CD4+^	% CAR+ cells that were CCR7-neg CD45RA+*	% CAR+ cells that were CCR7-neg and CD45RA-neg*	% CAR+ cells that were CCR7+ and CD45RA-neg*	% CAR+ cells that were CCR7+ and CD45RA+*
1	73	60	38	4.3	61	31	3.5
2	65	55	43	5.7	31	53	10
3	75	62	32	1.2	48	49	1.8
4	59	42	42	0.4	70	29	0.1
5	72	67	30	2.3	42	53	2.7
6	67	27	68	3.2	41	52	3.7
7	84	67	30	1.8	74	23	1.6
8	54	45	29	39	21	9.3	31
9	82	75	22	42	5.5	2.9	50
10	65	39	56	0.6	63	35	0.8
11	63	77	16	0.4	61	38	0.5
12	71	46	49	2	65	31	1.7
13	84	46	46	0.6	53	45	1
14	76	76	22	9	42	36	13
15	63	75	21	1	76	23	0.6

**%$CD3^+$ cell expressing the CAR was calculated by subtracting the %$CD3^+CAR^+$ events of untransduced T cells from the %$CD3^+CAR^+$ events of CAR-transduced T cells; CAR expression was determined by anti-Fab staining. The percentages of infused cells that were either $CD4^+$ or $CD8^+$ were determined on total infused $CD3^+$ cells. The sum of the percentages of $CD3^+CD4^+$ plus $CD3^+CD8^+$ cells does not equal 100% because some $CD3^+$ cells were either double negative T cells (CD4-negative, CD8-negative) or double positive T cells ($CD4^+CD8^+$). *CCR7 and CD45RA analysis was performed on $CD3^+CAR^+$ cells.

Supplemental Table 3

Peak serum cytokine levels during the 2 weeks after CAR T-cell infusion

Patient	Peak interferonγ (pg/mL)	Peak interleukin-6 (pg/mL)	Peak tumor necrosis factor (pg/mL)
1	165	882	40
2	43	206	0
3	61	201	17
4	426	104	14 (not increased from baseline)
5	396	2023	5
6	90	94	14
7	31	25 (only a 2-fold increase from baseline)	1
8	374	1049	33
9	108	242	11
10	81	36	0
11	53	29 (only a 4-fold increase from baseline)	0
12	243	39 (only a 3-fold increase from baseline)	0
13	54 (only a 2-fold increase from baseline)	20538	5
14	327	87	0
15	425	376	0

All cytokine levels were determined on patient serum by standard ELISA.

Except for the levels that are 5 pg/mL or less, unless otherwise noted, all peak cytokine levels were at least 5-fold higher than the pretreatment level.

Supplemental Table 4

Central nervous system tissues studied by quantitative PCR and found to lack CD19 RNA

Frontal Lobe
Temporal Lobe
Occipital Lobe
Parietal Lobe
Paracentral Gyrus
Postcentral Gyrus
Olfactory Bulb
Thalamus
Corpus Callosum
Hypothalamus
Amygdala
Hippocampus
Caudate
Putamen
Substantia Nigra
Pituitary Gland
Cerebellum Grey Matter
Cerebellum White Matter
Cerebellum Vermis
Nucleus Accumbens
Pons
Medulla
Spinal Cord
Choroid Plexus

Supplemental Table 5

Brain tissues stained by immunohistochemistry and found to not express CD19

Tissue stained
Cerebrum, frontal lobe
Cerebrum, apical lobe
Cerebrum, occipital lobe
Cerebrum, temporal lobe
Cerebrum, midbrain
Pons
Medulla oblongata
Thalamus opticus
Cerebellum
Hippocampus
Callositas
Optic nerve
Spinal cord

Supplemental Table 6

Blood B-cell counts of the 3 patients with normal levels of polyclonal B cells pretreatment

Patient	Pretreatment B-cell count	B-cell count 4 months post-treatment
1	291	0
10	386	1
15	142	1

All other patients had either undetectable blood B cells or nearly all CLL B cells prior to anti-CD19 CAR T cell infusion, so normal B-cell depletion was not evaluable.

Supplemental Table 7

Blood lymphocyte counts on the day of CAR T-cell infusion

Patient	Blood lymphocyte count on the day of CAR T-cell infusion (units are x1000/μL)
1	0
2	0.02
3	0.03
4	0
5	0.02
6	0
7	0
8	0
9	0.01
10	0.02
11	0.02
12	0.01
13	0.04
14	0.03
15	0.01

Supplemental Table 8

Prior treatments received by each patient before treatment on the anti-CD19 CAR T-cell trial

Patient	Prior treatments
1	%R, ^R-CVP, !R-CHOP, NCI anti-CD19 CAR clinical trial (current report is re-treatment)
2	R-CHOP, @R-ICE, Methotrexate+ARA-C, X-ray therapy
3	chlorambucil+prednisone, FC
4	R-CHOP, R-ICE, CODOX-M+bortezomib
5	fludarabine, fludarabine+mitoxantrone, pulse methylprednisolone, NCI FC+anti-CD19 CAR trial (current report is re-treatment)
6	*FC+rituximab
7	R-CHOP, R-ICE+lenalidomide, phase I clinical trial, X-ray therapy, R+cytarabine+etoposide
8	R-CHOP, R-ICE, brentuximab, R-high-dose ara-c, lenalidomide+rituximab, ^^R-GDP, X-ray therapy, 3 different phase I clinical trials
9	^^^R-EPOCH, R-ICE, gemcitabine+oxaliplatin+rituximab
10	R-CHOP, X-ray therapy, R-ICE, high-dose chemotherapy+autologous stem cell transplant
11	fludarabine, fludarabine+R, bendamustine+R-2 different times, @@hyper-CVAD-2 different times, R-ICE, X-ray therapy, R+methylprednisolone, R+cisplatin+gemcitibine+dexamethasone, methotrexate+ara-C, R+Ara-C
12	R-CHOP, R-ICE, X-ray therapy
13	Fludarabine-2 different times, R, bendamustine+R
14	R-CHOP, ##R-ESHAP
15	R-CHOP, R-ICE, high-dose chemotherapy followed by autologous stem cell transplantation

!R-CHOP=rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone

^R-CVP=rituximab, cyclophosphamide, vincristine, prednisone

@R-ICE=rituximab, ifosphamide, carboplatin, and etoposide

#R-DHAP=rituximab, dexamethasone, high-dose ara-c, and cisplatin

%R=rituximab

*FC=fludarabine plus cyclophosphamide

**CODOX-M=cyclophosphamide, cytarabine, vincristine, doxorubicin, methotrexate

^^R-GDP=rituximab, gemcitabine, dexamethasone, cisplatin

^^^R-EPOCH=rituximab, etoposide, prednisone, vincristine, cyclophosphamide, doxorubicin

@@hyper-CVAD=cyclophosphamide, vincristine, doxorubicin, dexamethasone

##R-ESHAP=etoposide, methylprednisolone, high-dose ara-c, cisplatin

Supplemental Table 9

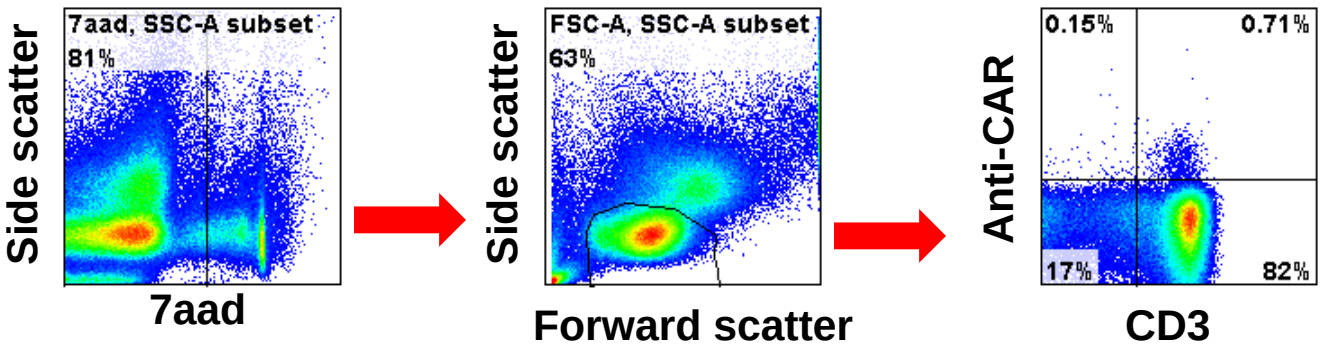
Peak percentage of CD3+CAR+ T cells determined by flow cytometry and peak absolute numbers of CAR+ T cells determined by both flow cytometry and quantitative PCR

Patient	Peak absolute number of CAR+ T cells (cells/μL) determined by qPCR	Peak absolute number Of CAR+ T cells (cells/μL) determined by flow cytometry	Peak percentage of Blood CD3+ cells that expressed the anti-CD19 CAR as determined by flow cytometry
1	115	119	8
2	60	163	33
3	86	21	3
4	476	375	51
5	166	45	27
6	28	22	2
7	9	2	2
8	14	4	3
9	56	30	49
10	33	108	28
11	22	55	19
12	33	28	11
13	50	62	23
14	35	25	42
15	777	1056	31

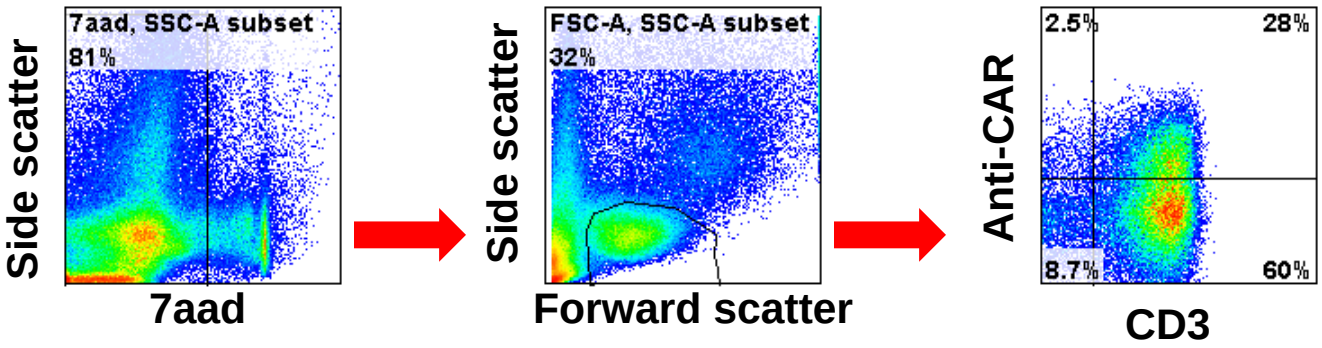
The peak absolute number of CAR+ T cells was first determined by qPCR as described in the Supplemental Methods then flow cytometry was performed on PBMC from the time-point of the peak in absolute number by using an anti-CAR monoclonal antibody as described in the Supplemental Methods. The absolute number of CAR+ T cells was determined by flow cytometry as described in the Supplemental Methods.

Supplemental Figure 1

Patient 15 pretreatment PBMC



Patient 15 PBMC collected 8 days after CAR T-cell infusion



The gating strategy used for anti-CAR monoclonal antibody staining is shown. Dead cells were excluded with 7aad staining. A lymphocyte gate was drawn, and CD3 and anti-CAR staining of total lymphocytes was shown. The example shown is Patient 15 as. The monoclonal anti-CAR antibody used in Figures 3 and 5 was also used in this staining.