CNS infiltration by zamtocabtagene autoleucel tandem CD20/CD19 CAR T cells leading to complete remission in a patient with primary CNS lymphoma

by Nancy M. Hardy, Jean A. Yared, Tim Luetkens, Haroon Ahmad, Rediet Mulatu, Daniel Yamoah, Xiaoxuan Fan, Samuel Weeks, Sophia A. Bredar, Ashley Gelin, Jillian M. Baker, Kenneth A. Dietze, Aerielle Matsangos, Ariel Fromowitz, Johanna Theruvath, Louisa Wirthlin, Remigiusz Kaleta, Anna Wijatyk, Rima Koka, Michael E. Kallen, Kim G. Hankey, Aaron P. Rapoport and Djordje Atanackovic

Case Report

CNS infiltration by zamtocabtagene autoleucel
tandem CD20/CD19 CAR T cells leading to complete remission
in a patient with primary CNS lymphoma

Nancy M. Hardy[1,2], Jean A. Yared[1,2], Tim Luetkens[1,2,3], Haroon Ahmad[1,4], Rediet Mulatu[1,2], Daniel Yamoah[1,2], Xiaoxuan Fan[1,3,5], Samuel Weeks[1,5], Sophia A. Bredar[1,3], Ashley Gelin[1,3], Jillian M. Baker[3], Kenneth A. Dietze[3], Aerielle Matsangos[1,2], Ariel Fromowitz[1,2], Johanna Theruvath[6], Louisa Wirthlin[6], Remigiusz Kaleta[6], Anna Wijatyk[6], Rima Koka[7], Michael E. Kallen[7], Kim G. Hankey[1,2], *Aaron P. Rapoport[1,2], *Djordje Atanackovic[1,2,3]

[1]Department of Medicine, University of Maryland School of Medicine, Baltimore, MD, USA
[2]Transplant and Cellular Therapy Program, University of Maryland Greenebaum Comprehensive Cancer Center, Baltimore, MD, USA
[3]Department of Microbiology and Immunology, University of Maryland, Baltimore, MD, USA
[4]Department of Neurology, University of Maryland School of Medicine, Baltimore, MD, USA
[5]University of Maryland Greenebaum Comprehensive Cancer Center, Baltimore, MD, USA
[6]Miltenyi Biomedicine Inc., Gaithersburg, MD, USA
[7]Department of Pathology, University of Maryland School of Medicine, Baltimore, MD, USA

*Both authors contributed equally

DECLARATIONS

Conflict of Interest
T.L. receives a salary from AbbVie. The other authors declare that they do not have any competing interests.

Authors' contributions
N.M.H. and J.A.Y., A.P.R. designed the study, enrolled patients, analyzed the data, and wrote the manuscript. T.L. analyzed the data, prepared figures, and wrote the manuscript. H.A., A.F., K.G.H. analyzed data and wrote the manuscript. R.M., D.Y., X.F., J.M.B., K.A.D., A.M., R.K., M.E.K. processed samples, performed experiments, analyzed the data, and wrote the manuscript. S.A.B. and A.G. performed experiments. D.A. designed the study, analyzed the data, made figures, and wrote the manuscript.

Corresponding author:
Djordje Atanackovic, MD
Director, Cancer Immunotherapy
Medical Director, Fannie Angelos Cellular Therapeutics GMP Laboratory
University of Maryland Greenebaum Comprehensive Cancer Center
Bressler Research Building, Room 9-011
655 W. Baltimore Street
Baltimore, MD 21201
Email: datanackovic@som.umaryland.edu

Data Sharing Statement:

The data supporting the findings of this study are available from the corresponding author upon reasonable request. De-identified clinical and experimental data may be shared for non-commercial, academic research purposes, subject to institutional and ethical approval. Data sharing will comply with all applicable data protection and privacy regulations.

CD19-directed chimeric antigen receptor (CAR) T cell (CAR-T) therapy is a well-established treatment for B-cell malignancies, but accessibility, toxicities, lack of persistence, modest anti-tumor activity, restricted trafficking and tumor antigen escape are among its limitations [1]. Concern for excessive neurotoxicity led to exclusion of patients with central nervous system (CNS) involvement from clinical trials, and primary CNS lymphoma (PCNSL) is an exclusion on the approved products' labeling. PCNSL is a highly aggressive lymphoma, with a favorable response to initial chemotherapy/radiation, but compared with lymphomas outside the CNS, relapses are common, and survival is inferior. Moreover, there is a lack of approved standard care beyond first-line therapy and the prognosis for these PCNSL patients remains poor [2].

In approximately one third of all B cell lymphoma patients, resistance to CAR-T and relapses are accompanied by CD19 downregulation. Preclinical evidence suggests that dual antigen-targeting may overcome this problem [3]. To address antigen escape, the investigational anti-CD20/anti-CD19 CAR-T product MB-CART2019.1 (zamtocabtagene autoleucel; zamto-cel) was designed. Zamto-cel is a dual-targeting tandem-CAR construct with scFv regions of anti-CD19 and anti-CD20 linked in sequence by a flexible interchain linker, followed by CD8, 4-1BB and CD3 zeta domains [4]. Our group and others have evaluated zamto-cel in a pivotal phase II clinical trial (DALY II USA/MB-CART2019.1; NCT04792489) for the treatment of DLBCL patients who received at least 2 lines of treatment [5]. In addition to addressing antigen escape/relapse zamto-cel is non-cryopreserved with a vein-to-vein time of 14 days, improving cell yield and potency. Here we present a patient with recurrent leptomeninges-only PCNSL, ineligible for enrollment into the DALY II trial due to lack of a >1cm measurable lesion, whom we treated with zamto-cel under single-patient IND.

A 75 years-old male patient with a 12-year history of Parkinson's Disease presented in July of 2022 with memory loss and dyspraxia. Magnetic resonance imaging (MRI) showed new leptomeningeal enhancement; cerebrospinal fluid (CSF) flow cytometry identified a monoclonal,

kappa-restricted B-cell population expressing CD19, CD20, and dim CD11c. Investigational treatment with 6 cycles of TEDDI-R (temozolomide, etoposide, doxorubicin HCl liposome, dexamethasone, ibrutinib, and rituximab) resulted in clinical improvement and clearance of the CSF. However, MRI showed persistent medio-frontal and parietal leptomeningeal enhancement. For 15 months MRIs remained stable and CSF was negative for tumor cells, however, in June 2024 kappa-restricted monoclonal B cells reappeared in the CSF, with strong expression of CD19 (Figure 1C) and low CD20 expression, consistent with lymphoma progression. The patient reported no new symptoms, but MRI showed medial frontal and parietal meningeal enhancement. Importantly, a PET/CT did not show any signs of systemic disease. Under a spIND the patient received lymphodepleting chemotherapy with fludarabine/cyclophosphamide and tandem CD20-CD19-directed non-cryopreserved CAR-T (Figure 1B) at a dose of $2.5x10^6$ cells / kg in August of 2024. The infused CAR-T product showed a $CD4^+/CD8^+$ ratio of 3.6 and a transduction efficiency of 29%. The patient was also enrolled into the Institutional Review Board (IRB)-approved protocol 2043GCCC (IRB HP-00091736) for the immunomonitoring of patients following CAR-T treatment and provided written informed consent.

One day after receiving the zamto-cel infusion, the patient developed immune cell associated neurotoxicity syndrome (ICANS) Grade 2 with a temporary drop in his Immune Effector Cell-Associated Encephalopathy (ICE) score to 6/10 (Figure 1E). He also developed Grade 1 cytokine release syndrome (CRS). For concurrent ICANS and CRS (Figure 1E), tocilizumab and dexamethasone were administered intravenously. Over the next few hours, the CRS resolved (Figures 1A+3D) and his ICE score returned to 10/10 on day+2.

On day+7 the patient showed a marked increase in zamto-cel CAR-T in the peripheral blood (PB) (Figure 1A+D). The CAR-T were primarily composed of $CD4^+$ and $CD8^+$ T cells with a comparably smaller proportion showing a $CD4^+/CD8^+$ double-positive phenotype (Figure 1A). Most of the CAR-T in the periphery were central memory (CM)-type T cells with a smaller

subset of effector memory (EM)-type T cells. PB CAR-T included a much smaller percentage of less-differentiated stem cell memory (SCM) T cells than normal non-CAR-T from the same timepoint (Figure 3A).

The patient's CSF from day+7 post CAR-T treatment, showed zamto-cel CAR-T had also infiltrated the patient's CNS (Figure 1D). On that day, the patient had a CSF white blood cell count (WBC) of 4/µL with 76% lymphocytes. Compared to CAR-T in the PB, the CNS-infiltrating CAR-T showed a much higher proportion of CD4$^+$ cells and lower numbers of CD8$^+$ T cells (Figure 3A). In addition, the patient's CNS-infiltrating CAR-T showed a higher number of CM-type cells versus the same CAR-T in the PB (Figure 3A). Interestingly, the unique subpopulation of CD4$^+$/CD8$^+$ double-positive CAR-T was detectable in both the PB and the CNS, however, in the CNS a higher proportion of these cells expressed an EM phenotype (Figure 3C).

At the time of peak CAR-T expansion we evaluated the response. Importantly, at day+7 post-CAR-T the patient showed a complete clearing of the CSF from malignant B cells by cytology and flow cytometry. To identify mechanisms underlying the substantial CNS infiltration by CAR-T and the concurrent eradication of tumor cells, we investigated the patient's cytokine/chemokine milieu in the PB and the CSF. We found that in the PB, and even more so in the CNS, the CAR-T infusion resulted in marked increases in IP-10 (Figure 3D). However, only in the CSF, high levels of this chemokine were present pre-treatment (Figure 3D).

Over the following week, CAR-T numbers declined but they were still detectable at day+14 and day+21 in both the PB and the CSF (Figure 1A+D). On day+14, the patient had a CSF WBC of 6/µL with 88% lymphocytes. Interestingly, the proportion of CNS CD4$^+$/CD8$^+$ double-positive CAR-T doubled (Figure 3C). To understand CAR-T persistence in the CNS even after achieving an initial response, we investigated (Figure 2) factors involved in CAR-T survival/persistence. We found that the CAR-T response in the PB was associated with a strong expression of CD27, an established enhancer of CAR-T activation/persistence [6], which peaked on day+7 (Figure 2A).

We did not detect expression of exhaustion markers on the CAR-T such as PD-1 and LAG-3 except TIM-3 which showed a low-level expression on both day+7 and day+14 (Figure 2B). Interestingly, we observed an upregulation of CD127 (IL-7Rα), which is crucial for survival and protection from exhaustion of long-lived memory T cells [7, 8], on the patients CD4$^+$ and CD8$^+$ PB CAR-T compared to non-CAR-T from the same day (Figure 2C). Subsequent CSF evaluations (days 15, 50, 72 and 190) remained clear of lymphoma, an MRI performed on day+37 (Figure 1E) showed resolution of parafalcine enhancement (Figure 3 E+F), and subsequent imaging, including 6 months post treatment, showed continued remission with complete return to pre-lymphoma functionality and cognition. In addition, all subsequent PET/CTs confirmed continuous absence of systemic disease.

A prerequisite for CAR-T response is homing to the target organ and tumor infiltration. We demonstrated substantial trafficking across the blood-brain barrier and CNS infiltration by the CAR-T by day+7 post intravenous infusion. As in our previous report on lymphoma patients with secondary CNS involvement (SCNSL) treated with standard-of-care CD19 CAR-T [9], this resulted in local enrichment primarily of central memory CD4$^+$ T cells.

We observed CNS infiltration by a unique CD4$^+$/CD8$^+$ CAR-T subpopulation with stronger EM phenotype than the same cells in the PB. One week later the proportion of CNS CD4$^+$/CD8$^+$ had doubled, arguing for a specific CNS enrichment and possible role of these cells in eradicating the lymphoma. Some studies have indicated unique tumor-targeting properties of CD4$^+$/CD8$^+$ T cells [10] and possibly a role in promoting autoimmune phenomena [11] and Human T cell leukemia virus type 1 (HTLV-1)-associated CNS disease [12]. Importantly, a recent study identified a CD4$^+$/CD8$^+$ double-positive T cell population in allo-HCT recipients where they predicted graft-versus-host disease (GVHD) [13]. Overall, we consider it possible that in CNS lymphoma this CAR-T subpopulation could play a role in mediating anti-tumor responses but also in promoting off-tumor toxicities.

To identify mechanisms underlying CAR-T CNS infiltration, we investigated the patient's local cytokine/chemokine milieu, observing marked increases in IP-10 following CAR-T infusion. This agreed with our previous study in SCNSL indicating that IP-10 may contribute to CNS recruitment of activated CAR-T, possibly through its receptor CXCR3 as in the case of T cell recruitment during neuroinflammation. Interestingly, CXCR3 is abundantly expressed on CNS-infiltrating T cells in multiple sclerosis patients [14] and coordinates CNS-directed T cell migration in response to its three ligands, CXCL9/CXCL10/CXCL11 [15]. However, only in the CSF, but not in the PB, high pre-treatment levels of IP-10 were present indicating that local growth of the lymphoma itself may initiate the production of this chemokine, i.e. by microglial cells.

In summary, we describe here the first case of a leptomeningeal PCNSL patient treated with tandem CD20-CD19-directed non-cryopreserved zamto-cel CAR-T. The patient tolerated the treatment well, showed a substantial expansion of CAR-T in the PB and had a deep and durable clinical response. One indispensable prerequisite for a clinical response to CAR-T is homing of the T cells to the target organ and we demonstrated trafficking across the blood-brain barrier and CNS infiltration by the CAR-T. In addition, we highlight mechanisms that potentially contributed to the CNS-homing of the CAR-T which may inform future strategies to optimize cellular immunotherapies, including multi-targeting CAR-T, for CNS lymphoma.

REFERENCES

1. Sterner RC, Sterner RM. CAR-T cell therapy: current limitations and potential strategies. Blood Cancer J. 2021;11(4):69.

2. Grommes C, DeAngelis LM. Primary CNS Lymphoma. J Clin Oncol. 2017;35(21):2410-2418.

3. Lupsilon and F, Whalen J, Song Y, et al. DLBCL cells emerge post CD19 CAR-T with cross-antigen resistance and a gene signature predictive of clinical CAR-T response. Blood Cancer Discov. 2025 Jun 27. doi: 10.1158/2643-3230.BCD-24-0176. [Epub ahead of print]

4. Schneider D, Xiong Y, Wu D, et al. A tandem CD19/CD20 CAR lentiviral vector drives on-target and off-target antigen modulation in leukemia cell lines. J Immunother Cancer. 2017;5:42.

5. Shah NN, Maziarz RT, Jacobson CA, et al. Tandem CD20-CD19-Directed Non-Cryopreserved CAR T Cells - Zamtocabtagene Autoleucel (Zamto-Cel) in Patients with Relapsed/Refractory Diffuse Large B Cell Lymphoma - Interim Results from a Phase 2 Pivotal Study (DALY II USA). Transplantat Cell Ther. 2025;31(2):S34.

6. Qin L, Cui Y, Yuan T, et al. Co-expression of a PD-L1-specific chimeric switch receptor augments the efficacy and persistence of CAR T cells via the CD70-CD27 axis. Nature Commun. 2022;13(1):6051.

7. Li L, Li Q, Yan ZX, et al. Transgenic expression of IL-7 regulates CAR-T cell metabolism and enhances in vivo persistence against tumor cells. Sci Rep. 2022;12(1):12506.

8. Tan JT, Dudl E, LeRoy E, et al. IL-7 is critical for homeostatic proliferation and survival of naive T cells. Proc Natl Acad Sci U S A. 2001;98(15):8732-8737.

9. Kline K, Luetkens T, Koka R, et al. Treatment of secondary CNS lymphoma using CD19-targeted chimeric antigen receptor (CAR) T cells. Cancer Immunol Immunother. 2024;73(3):45.

10. Alam MR, Akinyemi AO, Wang J, et al. CD4(+)CD8(+) double-positive T cells in immune disorders and cancer: Prospects and hurdles in immunotherapy. Autoimmunity Rev. 2025;24(3):103757.

11. Chang K, Na W, Liu C, et al. Peripheral CD4 (+)CD8 (+) double positive T cells: A potential marker to evaluate renal impairment susceptibility during systemic lupus erythematosus. J Biomed Res. 2022;37(1):59-68.

12. Maher AK, Aristodemou A, Giang N, et al. HTLV-1 induces an inflammatory CD4+CD8+ T cell population in HTLV-1-associated myelopathy. JCI Insight. 2024;9(1):e173738.

13. Hess NJ, Turicek DP, Riendeau J, et al. Inflammatory CD4/CD8 double-positive human T cells arise from reactive CD8 T cells and are sufficient to mediate GVHD pathology. Sci Adv. 2023;9(12):eadf0567.

14. Mahad DJ, Howell SJ, Woodroofe MN. Expression of chemokines in the CSF and correlation with clinical disease activity in patients with multiple sclerosis. J Neurol Neurosurg Psychiatry. 2002;72(4):498-502.

15. Loetscher M, Gerber B, Loetscher P, et al. Chemokine receptor specific for IP10 and mig: structure, function, and expression in activated T-lymphocytes. J Exp Med. 1996;184(3):963-969.

FIGURE LEGENDS

Figure 1: **CAR T cell expansion and persistence in a patient with primary CNS lymphoma post anti-CD20/anti-CD19 zamto-cel CAR T cells. (A)** Peripheral blood mononuclear cells (PBMCs) were isolated using density gradient centrifugation and analyzed immediately or cryopreserved until analysis. Total CAR T cell numbers and proportions of $CD4^+$, $CD8^+$, and $CD4^+/CD8^+$ double-positive CAR T cells after lymphodepleting chemotherapy (orange line) and CAR T cell infusion (black arrow). Proportions of the different CD19/CD20-targeted CAR T cells were determined in the peripheral blood of our patient at different timepoints using flow cytometry. CAR T cells were identified by staining of the CAR on the cell surface using a recombinant CD19 protein as CAR detection reagent (Miltenyi Biotec, cat# 130-115-965) and co-staining with anti-CD3 and other T cell markers. Samples were acquired using a Miltenyi MACSQuant Analyzer 10 Flow Cytometer. Analysis of flow cytometry data was performed using FlowJo software (BD Biosciences, San Jose, CA). The black dotted lines indicate the timepoints where CSF analyses took place. Administration of tocilizumab (toci; turquoise triangle) for CRS (purple line) is also indicated. Percentages of total CAR T cells, $CD4^+$, $CD8^+$ and $CD4^+/CD8^+$ are ishown on the left y-axis, while absolute numbers of CAR T cells per nL are shown on the right y-axis. **(B)** Structure of the MB-CART2019.1 (zamtocabtagene autoleucel) CAR. Zamto-cel CAR-T were manufactured in a closed system (CliniMACS Prodigy®) over 12 days from leukapheresis-derived $CD4^+$ and $CD8^+$ T cells. After enrichment, activation (CD3/CD28 with IL-7/IL-15), and lentiviral transduction with a CD19/CD20 CAR (4-1BB/CD3ζ signaling), cells were expanded and analyzed for phenotype, function, and final formulation. **(C)** Flow cytometric analysis of the PCNSL cells in the CSF prior to enrollment into our study. Gating was performed on cells with a blastoid morphology. Monoclonal B cells showed strong and homogenous expression of the CD19 target antigen on their surface. We did not determine CD20 expression as part of this analysis. and of **(D)** Dot plots (two upper rows) show percentages of CAR-

expressing T cells vs. all T cells in the patient's peripheral blood (PB; upper row) and cerebrospinal fluid (CSF; middle row), respectively, at different timepoints. To control for unspecific background staining a Fluorescence Minus One (FMO) control was used (lower row). **(E)** Timeline of events post CAR-T infusion.
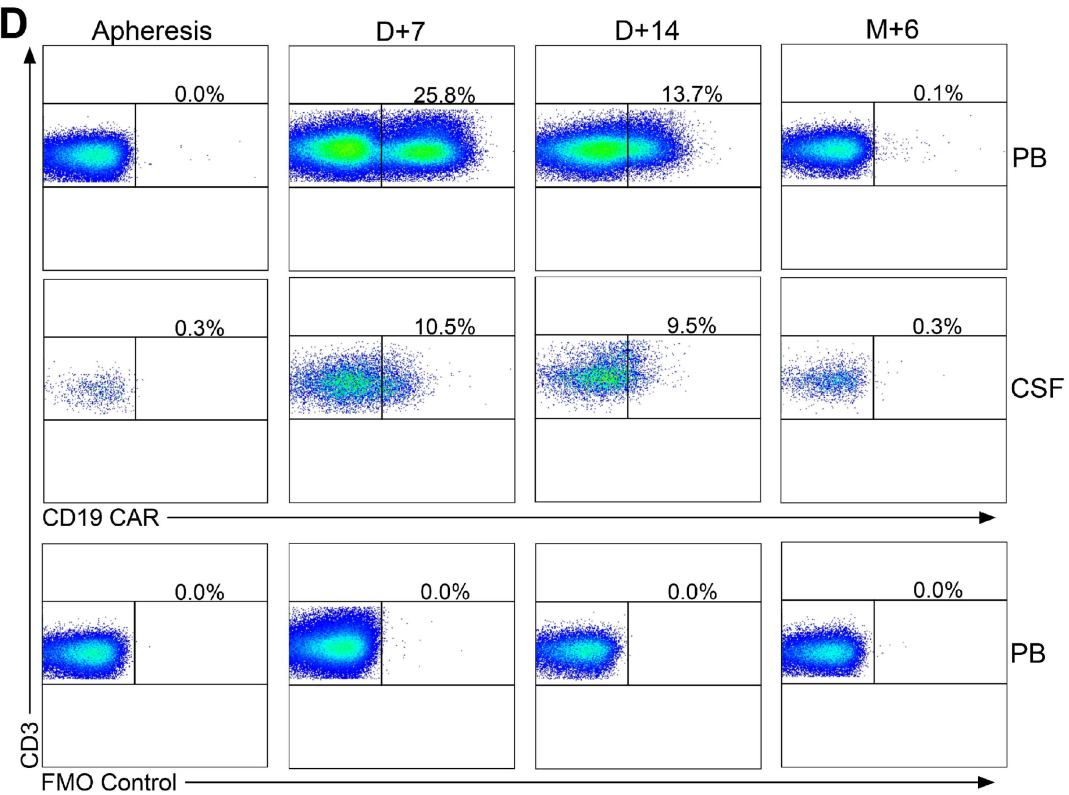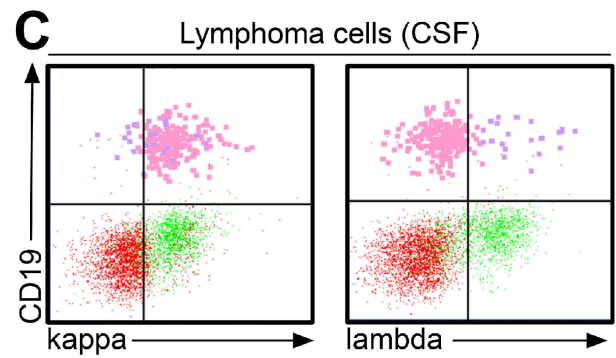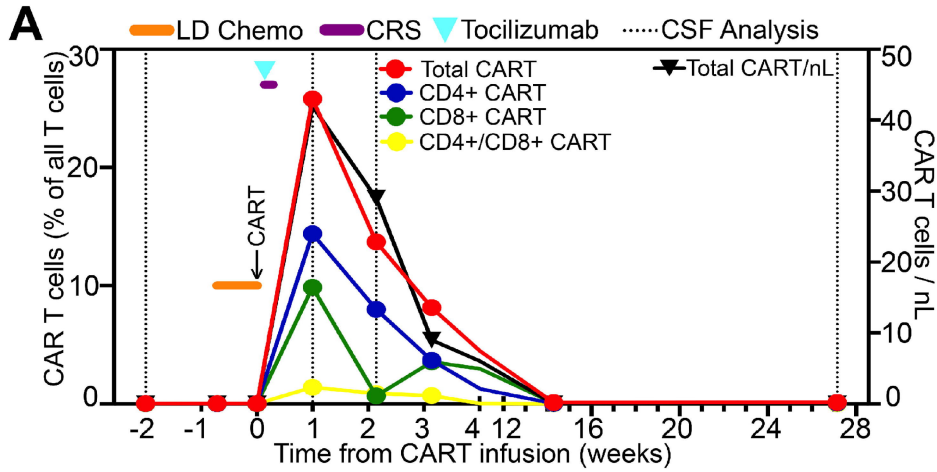
Figure 2: Factors potentially promoting persistence of the zamto-cel CAR-T in our PCNSL patient. (A) Surface expression levels of CD27 (Miltenyi, Cat#130-120-036) on PB CAR T cells (left column) from our patient compared to their own non-CAR-T (right column) on day+7 and day+14 post CAR-T treatment, respectively. To control for unspecific background staining an FMO control was used (gray histogram). **(B)** Surface expression levels of exhaustion markers PD-1 (Miltenyi, Cat#130-120-385), LAG-3 (Miltenyi, Cat#130-118-549), and TIM-3 (Miltenyi, Cat#130-119-781) on PB CD4$^+$ (upper row) and CD8$^+$ (lower row) CAR T cells from our patient on day+7 and day+14 post CAR-T treatment, respectively. To control for unspecific background staining an FMO control was used (gray histogram). **(C)** Surface expression levels of CD127 (IL-7Rα; Miltenyi, Cat#130-113-413) on PB CD4$^+$ or CD8$^+$ CAR T cells on day+7 post CAR-T infusion from our patient compared to their own non-CAR-T. To control for unspecific background staining an FMO control was used (gray histogram).

Figure 3: CNS infiltration by effector memory-type CART cells producing an inflammatory cytokine signature

Analyses of CD19/CD20-targeted CAR T cell numbers in the PB and CSF from **(A)** d+7 and **(B)** d+14 after CAR-T infusion were performed by flow cytometry following co-staining with anti-CD3 (Miltenyi, Cat#130-113-138) and CD19 CAR detection reagent (Miltenyi, Cat#130-129-550) which represents a fluorescent, full-length, recombinant CD19 protein binding to the CAR expressed on the cell surface. CAR T cell subpopulations in the PB and CSF were determined following staining with anti-CD4 (Miltenyi, Cat#130-113-230) and anti-CD8 (Miltenyi, Cat#130-

110-681) monoclonal antibodies. Different CAR-T memory subpopulations (SCM = cell-like memory; CM = central memory; EM = effector memory; EMRA = terminally differentiated effector memory) in the PB and CSF were identified by flow cytometry using co-staining with anti-CD45RA (Miltenyi, Cat#130-117-743) and anti-CD62L (Miltenyi, Cat#130-113-621) monoclonal antibodies **(C)** Percentages of CD4$^+$/CD8$^+$ double-positive CAR T cells out of all CAR-T (upper half) and composition of memory T cell subtypes of CD4$^+$/CD8$^+$ double-positive CAR T cells (lower half) in the PB vs. CSF on d+7 and d+14 post CAR-T infusion, respectively. **(D)** Serum (left) and CSF (right) concentrations of 22 cytokines/chemokines were measured over time in our patient using CodePlex Secretome technology. Cytokine/chemokine concentrations were quantified using the CodePlex Secretome Human Adaptive Immune Panel kit (IsoPlexis, cat. no. CODEPLEX-2L01). To carry out the CodePlex analysis, samples were added to a chip microchamber. The chip was then loaded into the Isolight reader (Isoplexis, Branfold, CT) and automated analysis of raw data was performed using IsoSpeak software (Isoplexis). Results are shown as absolute concentrations in pg/mL. Performing T1-weighted post-gadolinium contrast MRIs of the brain/spine (pre) and brain only (post), we followed two different groups of CNS target lesions **(E+F)** over time and observed a complete resolution following CAR T cell treatment. Red and green arrows, respectively, point to individual CNS manifestations of the relapsed PCNSL. Responses were assessed according to the guidelines of the International PCNSL Collaborative Group.

A — LD Chemo, CRS, Tocilizumab, CSF Analysis

Total CART, CD4+ CART, CD8+ CART, CD4+/CD8+ CART, Total CART/nL

CAR T cells (% of all T cells) vs Time from CART infusion (weeks)

B αCD20 (leu16) — αCD19 (FMC63) — CD8 — 41BB — CD3ζ

C Lymphoma cells (CSF)
CD19 vs kappa / lambda

D Apheresis | D+7 | D+14 | M+6
PB: 0.0% | 25.8% | 13.7% | 0.1%
CSF: 0.3% | 10.5% | 9.5% | 0.3%
CD19 CAR
PB: 0.0% | 0.0% | 0.0% | 0.0%
CD3 / FMO Control

E

Days from CAR-T	Date	Event
-14	08/13/2024	Apheresis
-5	08/22/2024	Start LD Chemo
0	08/27/2024	CAR-T infusion
1	08/28/2024	Start CRS / ICANS
2	08/29/2024	End CRS / ICANS
7	09/03/2024	
14	09/11/2024	
21	09/18/2024	
28	09/26/2024	
37	10/03/2024	MRI
90	12/05/2024	
180	03/05/2025	

A

CAR T | Non-CAR T

CD4+ / CD8+ — CD27

Legend: D+7 (red), D+14 (blue), FMO (grey)

B

CAR T cells | CAR T cells | CAR T cells

CD4+ / CD8+ — PD-1 | LAG-3 | TIM-3

Legend: D+7 (red), D+14 (blue), FMO (grey)

C

D+7

CD4+ / CD8+ — CD127

Legend: CART (green), Non-CART (yellow), FMO (grey)

Counts (y-axis)