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

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 ζ domains.[4] Our group and others have evaluated zamto-cel in a pivotal phase II clinical trial (DALY II USA/MB-CART2019.1; *clinicaltrials gov. Identifier: NCT04792489*) for the treatment of DLBCL patients who received at least two 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 >1 cm measurable lesion, whom we treated with zamto-cel under single-patient single-patient investigational new drug.

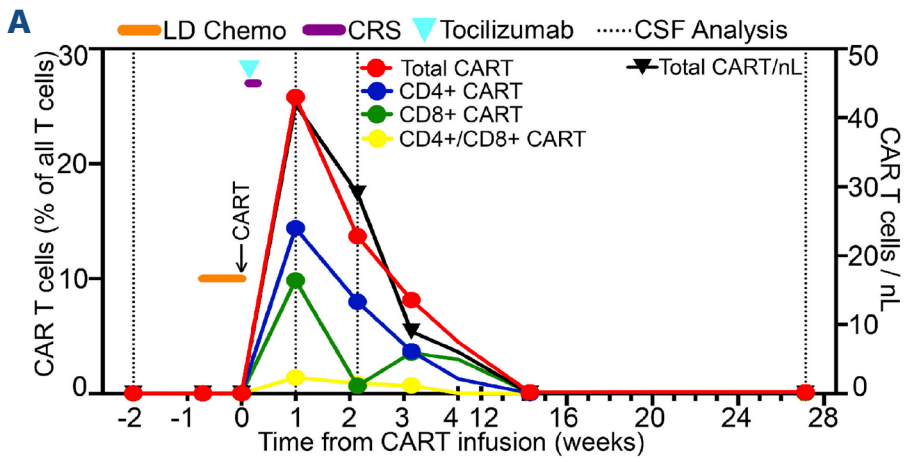
A 75-year-old male patient with a 12-year history of Parkinson's disease presented in July of 2022 with memory loss and dyspraxia. Magnetic resonance imaging (MRI) showed new leptomeningeal enhancement; cerebrospinal fluid (CSF) flow cytometry identified a monoclonal, κ-restricted B-cell population expressing CD19, CD20, and dim CD11c. Investigational treatment with six 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 MRI remained stable and CSF was negative for tumor cells, however, in June 2024 κ-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 positron emission tomography/computed tomography (PET/CT) did not show any signs of systemic disease. Under a single-patient investigational new drug the patient received lymphodepleting chemotherapy with fludarabine/cyclophosphamide and tandem CD20-CD19-directed non-cryopreserved chimeric antigen receptor (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.

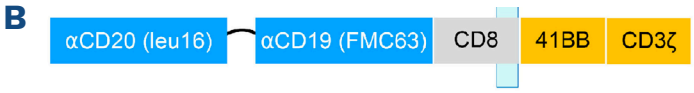
One day after receiving the zamto-cel infusion, the patient developed immune cell-associated neurotoxicity syndrome (ICANS) grade 2 with a temporary drop in his immune effector cell-associated encephalopathy (ICE) score to 6/10 (Figure 1E). He also developed grade 1 cytokine release syndrome (CRS). For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

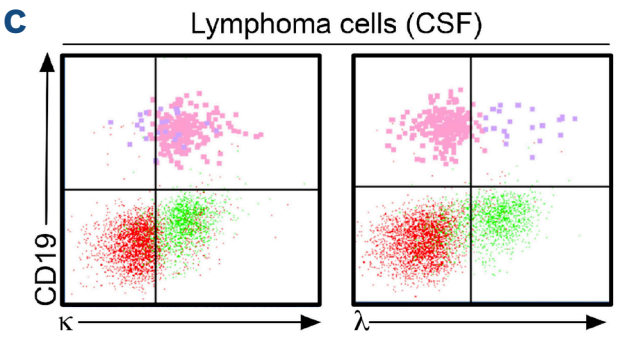
On day+7 the patient showed a marked increase in zamto-cel CAR T in the peripheral blood (PB) (Figure 1A, D). The CAR T were primarily composed of $CD4^+$ and $CD8^+$ T cells with a comparably smaller proportion showing a $CD4^+/CD8^+$ double-positive phenotype (Figure 1A). Most of the CAR T in the periphery 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 time point (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 $4x10^6$/L with 76% lymphocytes. Compared to CAR T in the PB, the CNS-infiltrating CAR T showed a much higher proportion of $CD4^+$ cells and

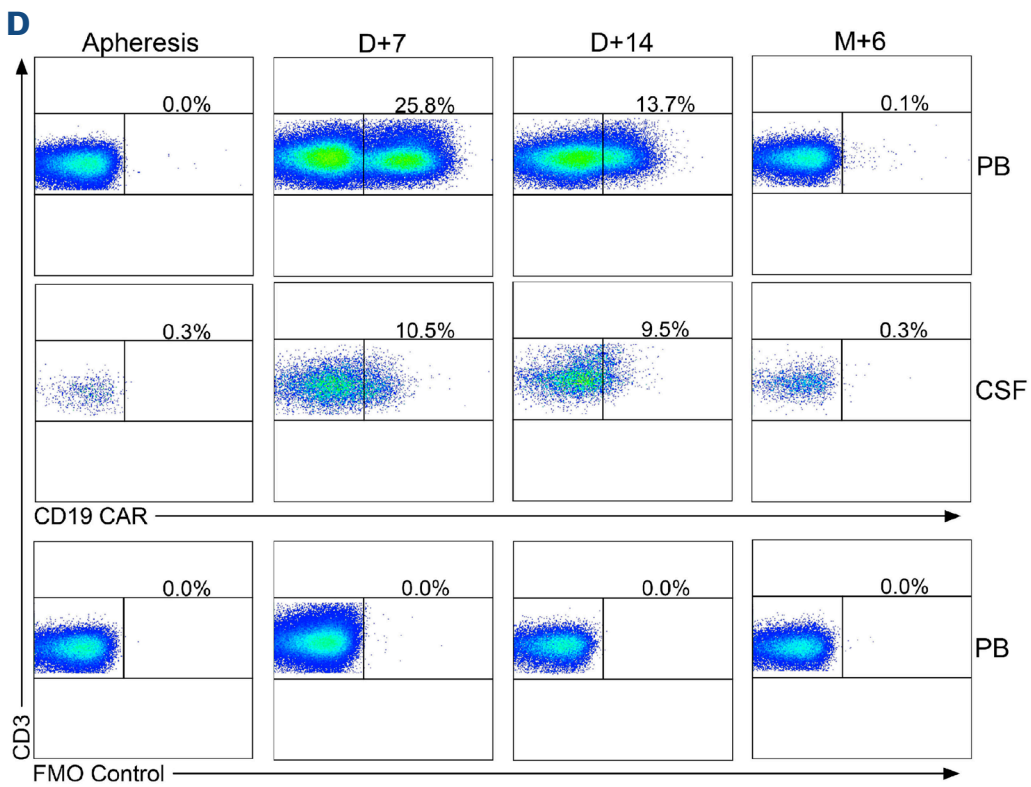
lower numbers of CD8⁺ T cells (Figure 1A). In addition, the patient's CNS-infiltrating CAR T showed a higher number of CM-type cells *versus* the same CAR T in the PB (Figure 3A). Interestingly, the unique subpopulation of CD4⁺/CD8⁺ double-positive CAR T was detectable in both the PB and the CNS, however, 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









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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	

Figure 1. CAR T-cell expansion and persistence in a patient with primary central nervous system lymphoma post anti-CD20/anti-CD19 zamto-cel CAR T cells. (A) Peripheral blood mononuclear cells (PBMC) were isolated using density gradient centrifugation and analyzed immediately or cryopreserved until analysis. Total chimeric antigen receptor (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 (PB) of our patient at different time points 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 time points where cerebrospinal fluid (CSF) analyses took place. Administration of tocilizumab (toci; turquoise triangle) for cytokine release syndrome (CRS) (purple line) is also indicated. Percentages of total CAR T cells, CD4⁺, CD8⁺ and CD4⁺/CD8⁺ are shown 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 [zamto-cel]) 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 primary central nervous system lymphoma (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. (D) Dot plots (2 upper rows) show percentages of CAR-expressing T cells *versus* all T cells in the patient's PB (upper row) and cerebrospinal fluid (CSF; middle row), respectively, at different time points. To control for non-specific background staining a fluorescence minus one (FMO) control was used (lower row). (E) Timeline of events post CAR T infusion. D: day; LD chemo: low-dose chemotherapy; MRI: magnetic resonance imaging; ICANS: immune effector cell–associated neurotoxicity syndrome.

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 6x10^6/L 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 significant 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, although TIM-3 did show 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 patient's 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 showed resolution of parafalcine enhancement (Figure 3E, 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/CT confirmed continuous absence of systemic disease.

A prerequisite for CAR T response is homing to the tumor target 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

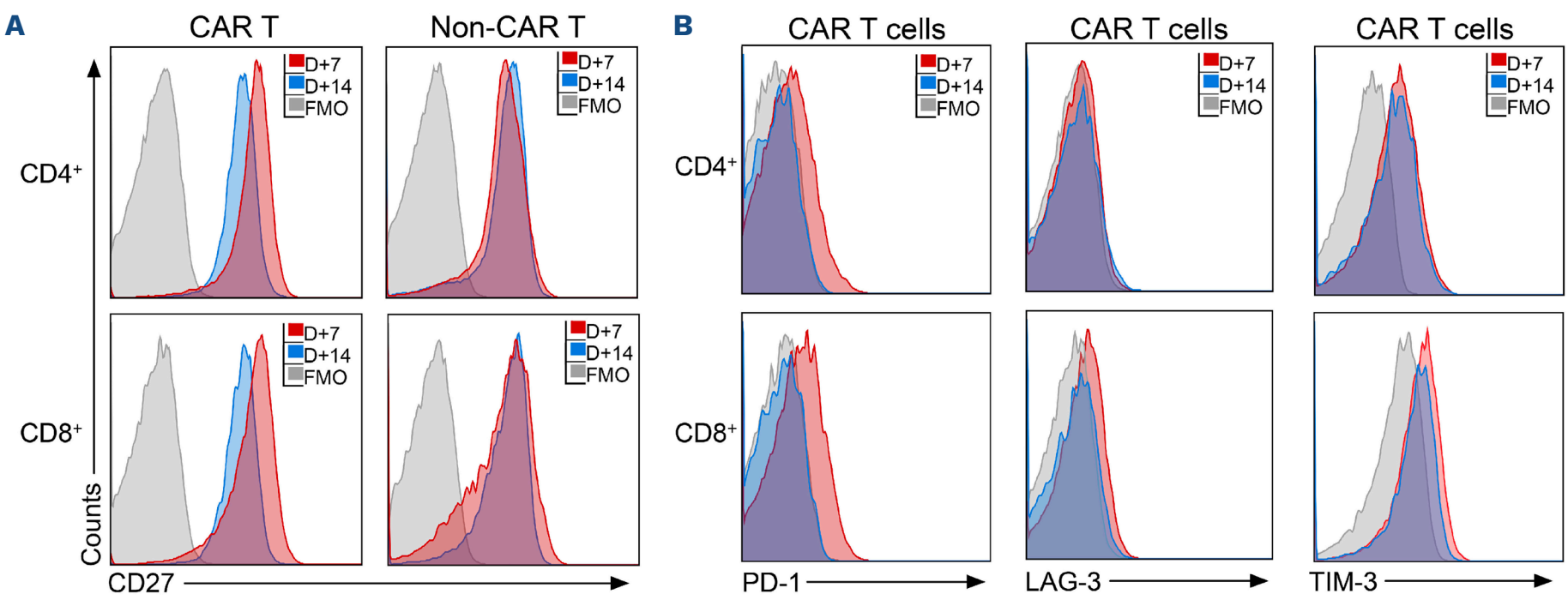
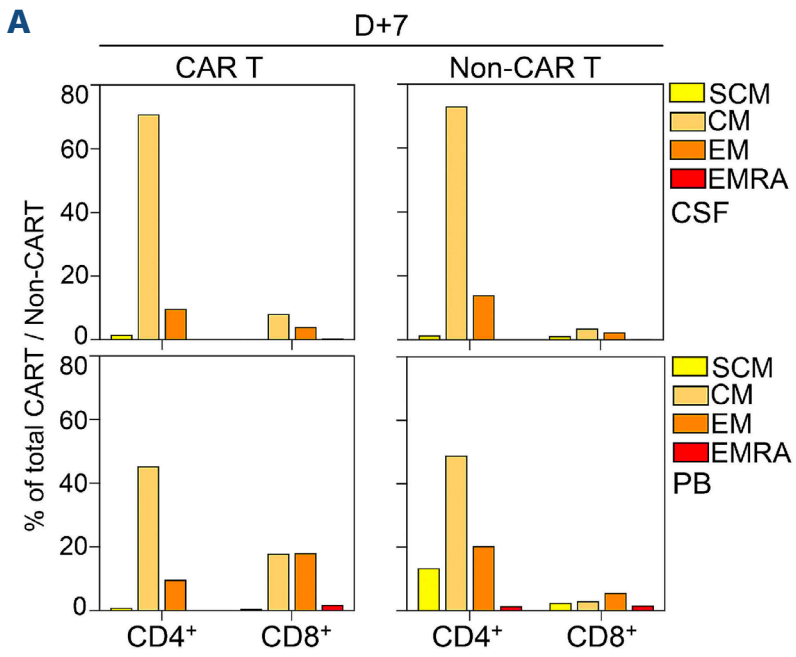
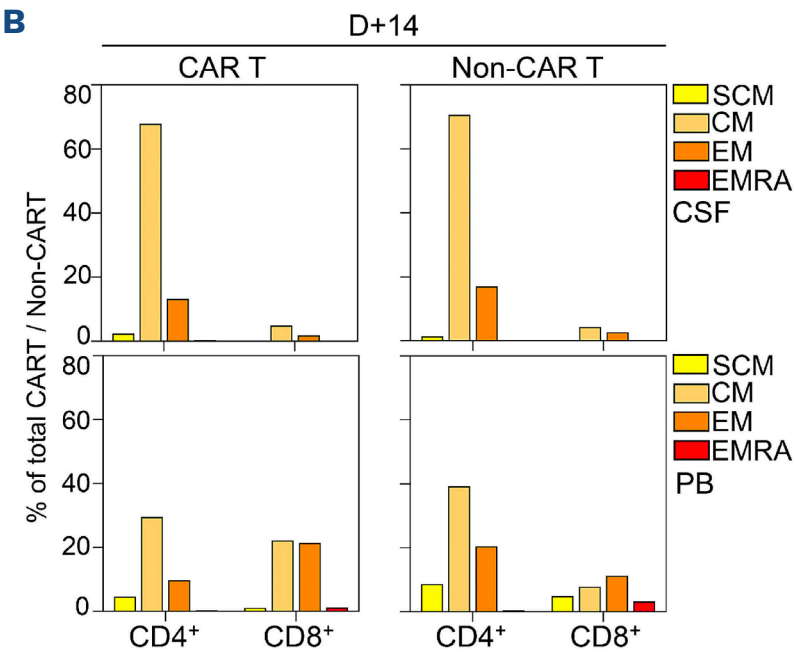
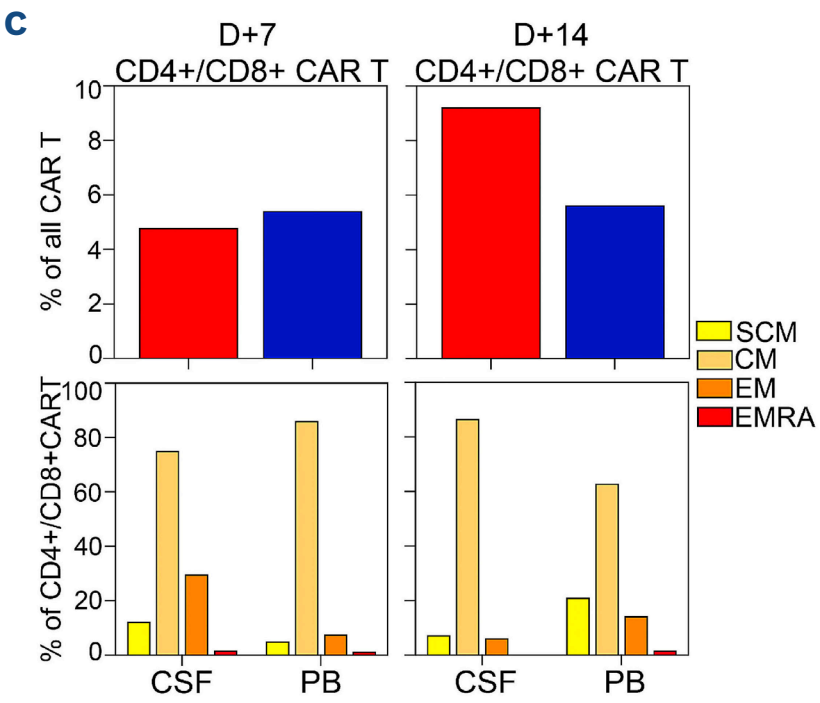
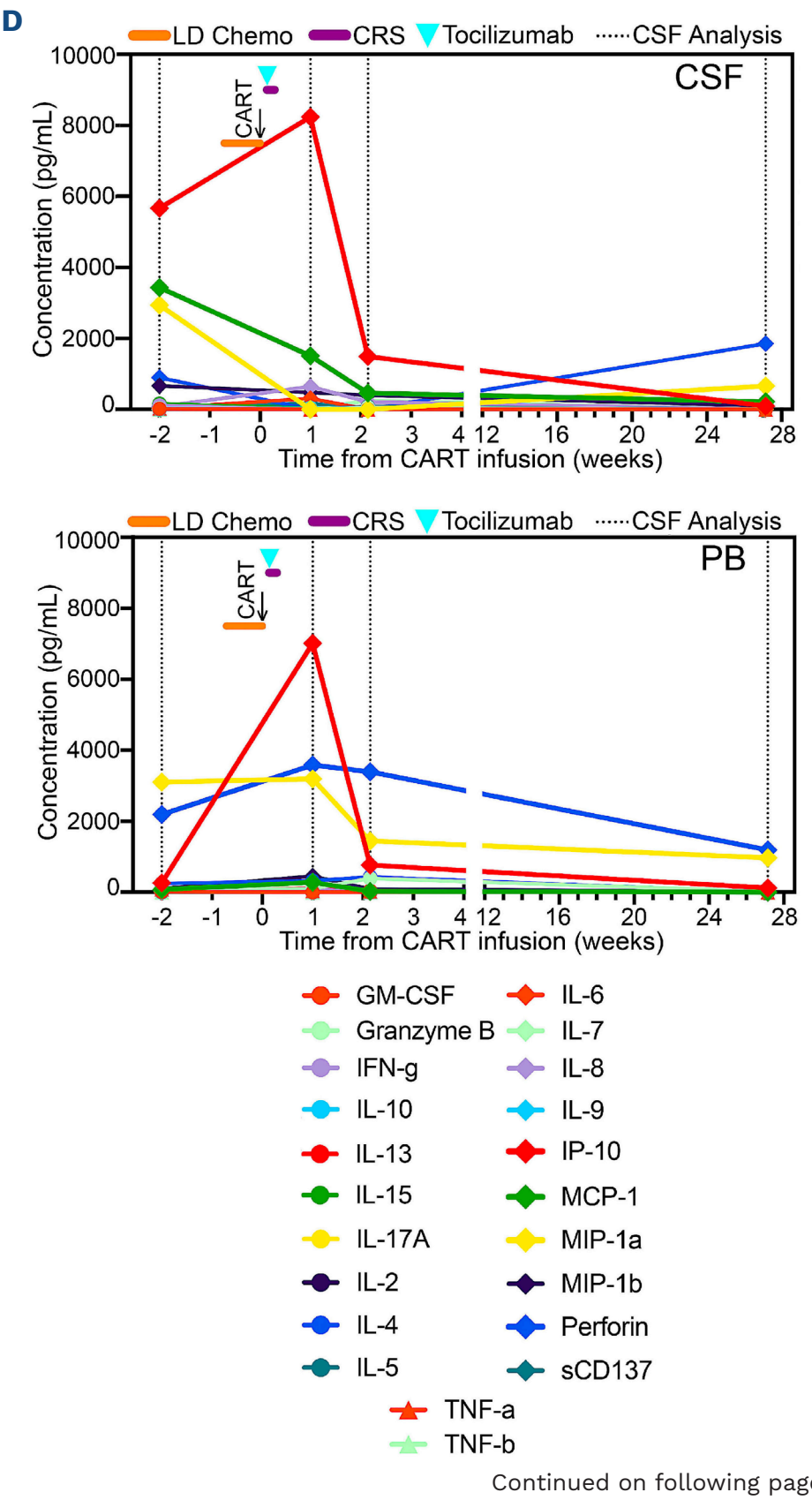


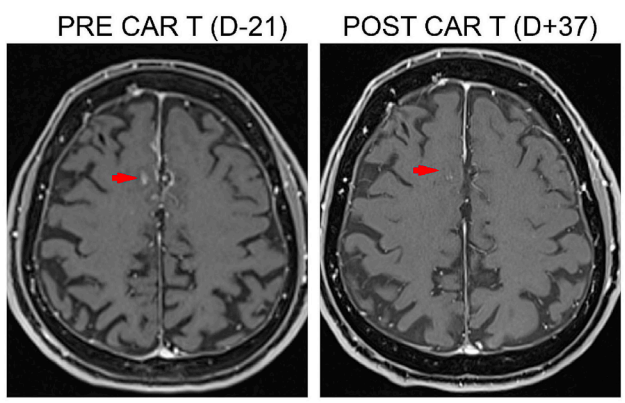
Figure 2. Factors potentially promoting persistence of the zamto-cel CAR T in our PCNSL patient. (A) Surface expression levels of CD27 (Miltenyi, Cat#130-120-036) on peripehral blood (PB) chimeric antigen receptor (CAR) T cells (left column) from our patient compared to their own non-CAR T cells (right column) on day+7 (D+7) and day+14 (D+14) post CAR T treatment, respectively. To control for unspecific background staining a fluorescence minus one (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 D+7 and D+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 D+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).











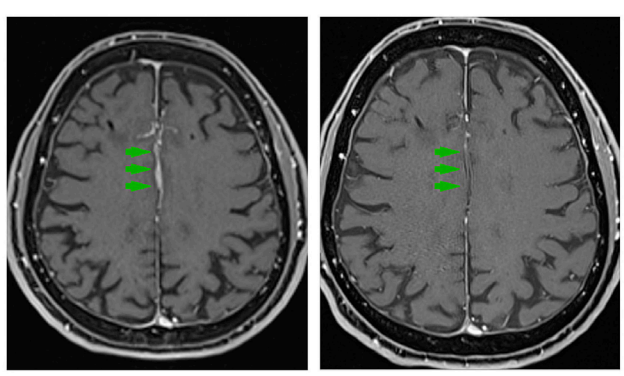


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Figure 3. Central nervous system infiltration by effector memory-type CAR T cells producing an inflammatory cytokine signature. Analyses of CD19/CD20-targeted chimeric antigen receptor (CAR) T-cell numbers in the peripheral blood (PB) and cerebrospinal fluid (CSF) from (A) day 7 (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: stem cell-like memory; memory; CM: central; EM: effector memory; TEMRA: terminally differentiated effector memory re-expressing CD45RA) 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 (blue) *versus* CSF (red) on D+7 and D+14 post CAR T infusion, respectively. (D) CSF (top) and serum (bottom) 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, catalog number 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 magnetic resonance imaging of the brain/spine (pre) and brain only (post), we followed 2 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 central nervous system lymphoma (PCNSL). Responses were assessed according to the guidelines of the International PCNSL Collaborative Group.

secondary CNS involvement treated with standard-of-care CD19 CAR T,[9] this resulted in local enrichment of primarily 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-associated CNS disease.[12] Importantly, a recent study identified a $CD4^+/CD8^+$ double-positive T-cell population in allogeneic stem cell transplantation recipients where they predicted graft-*versus*-host disease.[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 and 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 tumor target, 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.

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Disclosures

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Contributions

NMH, JAY, and APR designed the study, enrolled patients, analyzed the data, and wrote the manuscript. TL analyzed the data, prepared figures, and wrote the manuscript. HA, AF, and KGH analyzed data and wrote the manuscript. RM, DY, XF, JMB, KAD, AM, RK, and MEK processed samples, performed experiments, analyzed the data, and wrote the manuscript. SAB and AG performed experiments. DA designed the study, analyzed the data, made figures, and wrote the manuscript.

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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.

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