**The Cerebroventricular Environment Modifies CAR T Cells for Potent Activity against Both Central Nervous System and Systemic Lymphoma**

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**ABSTRACT**

Lymphomas with central nervous system (CNS) involvement confer a worse prognosis than those without CNS involvement, and patients currently have limited treatment options. T cells genetically engineered with CD19-targeted chimeric antigen receptors (CAR) are effective against B-cell malignancies and show tremendous potential in the treatment of systemic lymphoma. We aimed to leverage this strategy toward a more effective therapy for patients with lymphoma with CNS disease. NOD-scid IL2Rγnull (NSG) mice with CNS and/or systemic lymphoma were treated with CD19-CAR T cells via intracerebroventricular (ICV) or intravenous (IV) injection. CAR T cells isolated after treatment were rigorously examined for phenotype, gene expression, and function. We observed that CAR T cells infused ICV, but not IV, completely and durably eradicated both CNS and systemic lymphoma. CAR T cells delivered ICV migrated efficiently to the periphery, homed to systemic tumors, and expanded in vivo, leading to complete elimination of disease and resistance to tumor rechallenge. Mechanistic studies indicated that ICV-delivered CAR T cells are conditioned by exposure to cerebrospinal fluid in the ICV environment for superior antilymphoma activity and memory function compared with IV-delivered CAR T cells. Further analysis suggested that manipulating cellular metabolism or preactivating therapeutic CAR T cells with antigen ex vivo may improve the efficacy of CAR T cells in vivo. Our demonstration that ICV-delivered CD19-CAR T cells had activity against CNS and systemic lymphoma could offer a valuable new strategy for treatment of B-cell malignancies with CNS involvement.

**Introduction**

Primary central nervous system (CNS) lymphoma represents 2% to 3% of all primary brain tumors and 1% to 2% of all lymphoma diagnoses (1). Systemic lymphoma with secondary CNS involvement occurs in 10% to 30% of patients with diffuse large B-cell lymphoma (2). Surgery is not usually an option to treat primary or secondary CNS lymphoma due to the tendency of CNS lymphoma to be near critical structures and multicentric. Although new therapeutic approaches have improved survival, the disease still poses a challenge to manage and is associated with a poor prognosis (3–5). Thus, it is critical to identify novel therapeutic modalities to treat CNS lymphoma.

Chimeric antigen receptor (CAR) T cells targeting CD19 have produced tremendous clinical success in treating several B-cell lineage malignancies, but thus far have not been used specifically to treat CNS lymphoma. In all CD19-CAR T-cell trials to date, T-cell products have been administered intravenously (IV). CD19-CAR T cells can traffic to the cerebrospinal fluid (CSF) following systemic administration (6–9), where they may mediate activity against CNS lymphoma. Although neurotoxicity is a common and potentially life-threatening toxicity of CD19-CAR T cells, there is no evidence indicating that the presence of CAR T cells in CSF is directly related to neurotoxicity (10). Therefore, the goals of this project were to identify ways to limit the toxicity that results from systemic infusion of high doses of CAR T cells (11–13) and to improve activity against CNS lymphoma.

Recent studies show that regional intracerebroventricular (ICV) delivery of rituximab for CNS lymphoma is well-tolerated (14–16). In addition, ICV delivery of CAR T cells is safe and effective in patients with glioblastoma and in preclinical animal studies of glioblastoma and brain-metastatic solid tumors (17–19). Therefore, we hypothesized that ICV delivery of CD19-CAR T cells could hold promise as an effective therapeutic approach for both primary CNS lymphoma and systemic lymphoma with CNS involvement. Here, we have demonstrated that a single dose of ICV-delivered CD19-CAR T cells completely eradicated both CNS and systemic lymphoma in a NOD-scid IL2Rγnull (NSG) mouse model. Further, our mechanistic studies established that CD19-CAR T cells exposed to CSF in the ICV environment exhibited memory features and enhanced...
antilymphoma activity when compared with systemically administered CD19-CAR T cells.

Materials and Methods

Cell lines

Daudi (Burkitt lymphoma) and Jeko [mantle cell lymphoma (MCL)] cells were obtained in 2017 from the ATCC and maintained in RPMI 1640 (Irvine Scientific) supplemented with 10% heat-inactivated FCS (Hyclone), 2 mmol/L-glutamine (Irvine Scientific), and 25 mmol/L HEPES (Irvine Scientific; complete medium). The cells were passaged twice a week up to 10 passages after thawing. Both cell lines are reauthenticated after thawing by CD19-positive expression analyzed with flow cytometry after staining with antibody against CD19 (Catalog No. IM1285U; Beckman Coulter). Mycoplasma testing was done regularly in house using the Myco alert Mycoplasma detection Kit (Lonza, LT07–318). To generate tumor cell lines expressing enhanced green fluorescent protein (EGFP) and firefly luciferase (fluc), Daudi/Jeko cells were transduced with a lentiviral vector encoding EGFP-fluc at a multiplicity of infection (MOI) of 3 in complete medium. After expansion in complete medium for 14 days, GFP+ cells were sorted by FACS for >98% purity and expanded for in vivo experiments. OKT3–2A-Hygro_− pEK (gift from Andrew Rautabetch, City of Hope National Medical Center) contains the anti-human-CD3ε immunoglobulin gene, the 2A peptide sequence, and the hygromycin resistance gene within the pEK vector (20). Epstein–Barr virus–transformed lymphoblastoid cell lines (LCL) were made from peripheral blood mononuclear cells (PBMC) as previously described (21). OKT3–expressing LCLs in experiments using the rapid expansion method (REM; ref. 22) were generated by electroporating allogeneic LCLs with OKT3–2A-Hygro_pEK plasmid using the Amaxa Nucleofector I (Lonza) program T-20 according to the manufacturer’s instructions. Resulting cells were grown and passaged for 2 months in complete medium supplemented with 0.4 mg/mL hygromycin (Stratagene).

Antibodies and flow cytometry

Fluorochrome-conjugated antibodies against CD3 (clone Leu-4), CD4 (clone SK3), CD8 (clone RPA-T8), CD25 (clone M-A251), CD107a (clone H4A3), CD28 (clone CD28.2), CD62L (clone SK11), CD127 (clone HIL-7R-M21), CD161 (clone DX12), and streptavidin–CD107a (clone H4A3), CD28 (clone CD28.2), CD62L (clone SK11), CD4 (clone SK3), CD8 (clone RPA-T8), CD25 (clone M-A251), and CD14 were purchased from BioLegend. Antibodies against KLRG (clone 2F1), Tim3 (clone F38fluc lentiviral vector at MOI 2. EGFR+ CAR T cells were purified with positive selection on the AutoMACS. Following selection, isolated Tn/mem cells were activated, transduced, and maintained as previously described (25–27), and nontransduced T cells were used as control (mock) T cells. Where indicated, CAR T cells were cultured in human CSF (Lee Biosolutions) or modified (60 mg/dL glucose; 2.8 mEq/L potassium) RPMI (In vitrogen) for 48 hours at 37°C. To generate EGFP-fluc–expressing CD19-CAR T cells, T cells were activated and cotransduced with CD19-CAR lentiviral vector at MOI 1 and EGFP-fluc lentiviral vector at MOI 2. EGFR+ CAR T cells were purified with EGFR EasySep (Catalog No. 18309; StemCell Technologies, Inc.) following the manufacturer’s instructions, resulting in 60% to 70% GFP+ EGFR+ double-positive cells. To prime CAR T cells in vitro, 2 × 10⁶ CD19-CAR T cells were cocultured with irradiated (8,000 rads) Daudi cells at 1:10 (CAR:tumor) ratio in a 24-well plate for 1 hour at 37°C prior to infusion into mice.

Orthotopic brain and systemic lymphoma xenograft model

All animal experiments were performed under protocols approved by the City of Hope Institutional Animal Care and Use Committee using mice that were bred at our institutional animal facility. Six- to 10-week-old NOD/Scid IL2Rγcnull mice were injected intracranially and/or subcutaneously (SC) with EGFP-fluc–Daudi or -Jeko cells. Mice were treated with CAR T cells or nontransduced mock cells by ICV (19, 28) or IV infusion. Anesthetized mice were imaged using a Xenogen IVIS 100 series system. Photons from fluc+ tumor xenografts were quantified using the software program Living Image (Xenogen), and the bioluminescence signal was measured as total photon flux normalized for exposure time and surface area, expressed in units of photons per second per cm² per steradian.

Reverse transcriptase quantitative PCR and single-cell RNA sequencing analysis

CAR T cells were cultured in RPMI or human CSF for 48 hours, and RNA was extracted with RNeasy Kits (Qiagen) according to the
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Reverse transcriptase quantitative PCR (RT-qPCR) was performed using the RT Profiler PCR Array (SABiosciences) designed with 48 customized genes involved with CAR T-cell differentiation, survival, metabolism, and apoptosis (Supplementary Table S1; refs. 29, 30) and commercial primers provided by SABiosciences on CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Four control genes were used including housekeeping genes PPIA (Peptidylprolyl isomerase A) and RPL13A (Ribosomal protein L13a), control for reverse transcription, and positive PCR control. All samples passed the RNA quality control, PCR array reproducibility, RT efficiency, and genomic DNA contamination according to the manufacturer’s instructions. Data from duplicates were normalized by PPIA and analyzed by ΔΔCt method using RT2 Profiler PCR Array Web portal software (https://dataanalysis.qiagen.com/pcr/arrayanalysis.php). Hierarchical clustering analysis was performed in Cluster 3.0 using the average linkage clustering method, with genes demonstrating an absolute fold change >1.3. Results were visualized in Treeview 1.1.6r4.

Figure 1.
ICV-infused CAR T cells eradicate CNS lymphoma more efficiently than IV-infused CAR T cells. A, In our models of CNS lymphoma, NSG mice were injected intracranially (IC) with either EGFP+ fluc+ Daudi lymphoma cells or Jeok MCL cells. Following tumor engraftment, Tn/mem-derived CD19- or BAFF-R-CAR T cells were administered IV or ICV. For the CD19-CAR T-cell model, 3 x 10^6 cells and 1.3 x 10^6 cells were administered IV and ICV, respectively. For the BAFF-R-CAR T-cell model, equal numbers of cells (1 x 10^6) were administered for both IV and ICV conditions. B, For the CD19-CAR T-cell model, tumor burden, as measured in flux (photons/sec) by BLI, was evaluated once weekly. N = 5 mice per group in one experiment. Linear mixed models were used to compare logarithm-transformed flux of mice that received ICV versus IV CAR T cells over time; **, P < 0.01. C, For the BAFF-R-CAR T-cell model, Flux (photons/sec) was determined by measuring bioluminescence once per week. N = 5 mice per group in one experiment. Linear mixed models were used to compare logarithm transformed flux of mice that received ICV versus IV CAR T cells over time; **, P < 0.01. Survival of mice treated with ICV- versus IV-delivered BAFF-R-CAR T cells was analyzed by the log-rank test; **, P < 0.01. D and E, Blood was collected 105 days after CD19-CAR T-cell treatment and analyzed for expression of human CD45, CD8, CD4, and CAR (Erbitux) by flow cytometry. Data from a representative mouse for ICV- and IV-delivered CD19-CAR T cells are shown in D. Percentages (mean ± SD) of human T cells (hCD45+GFP-) and CD4+ and CD8+ cells for all mice are quantified in E. Gating was set based on isotype-matched monoclonal antibodies or streptavidin. Mean ± SDs from multiple mice/measurements are depicted.
For single-cell RNA sequencing (RNA-seq) analysis, cells harvested from bone marrow of mice treated with CAR T cells were resuspended at 700 to 1,200 cells/μL in 0.04% BSA (Sigma) in PBS. Single-cell RNA libraries were prepared according to the Chromium Single Cell 3′ Reagent Kits v3 User Guide using the Chromium single-cell Controller instrument (10× Genomics). Libraries were sequenced with a HiSeq 2500 (Illumina) with a depth of 50 to 100 k reads per cell. Raw sequencing data were processed using the 10x Genomics Cell Ranger pipeline (version 3.0) and aligned to hg19 and mm10 genome. Data normalization was done using “Seurat” package v3.0, with library scaling and log transformation. All samples were processed by 10x instrument in the same batch, thus no batch removal was done. Cells with mitochondrial read rate >10% and <200 detectable genes were considered low quality and were not used in the analysis. Normalized and scaled data were clustered using the top significant principal components of highly variable genes and visualized by the t-distributed stochastic neighbor embedding (t-SNE) algorithm. Cluster-specific markers were identified to generate heatmap. The dot plots and feature plots in the identified cell clusters were generated for specific T-cell populations.

**In vitro T-cell functional assays**

For cytokine analysis, CD19-CAR T cells (10⁶) that were expanded in either human CSF as described previously or complete medium were cocultured in 96-well tissue culture plates with 10⁵ Daudi cells in complete medium at 37°C. Supernatants were harvested 18 hours after stimulation and analyzed in triplicate by cytometric bead array using the Bio-Plex Human Cytokine 17-Plex Panel (Bio-Rad Laboratories). For degranulation assays, CAR T cells (10⁵) were cocultured with Dauidi cells (10⁵) in medium containing Golgi plug (Catalog No. 555029; BD Biosciences) at 1 μL/mL and 50 μL/200 μL CD107a-specific antibody for 6 hours at 37°C. Degranulation was determined by CD107a positivity using multicolor flow cytometry (see Antibodies and flow cytometry above).

**NBDG uptake assay**

NBDG fluorescence displays excitation/emission maxima of approximately 465/540 nm and can be visualized using a flow cytometer with a green/FTTC channel. Spleens were collected from tumor-bearing mice following CAR T-cell treatment and stimulated using the REM (22) for 2 cycles (14 days/cycle). Propagated cells were stained with an APC-conjugated human CD62L antibody (clone SK11; BD Biosciences) and cultured in 100 μmol/L 2-NBDG (Catalog No. N13195; Fisher Scientific) in PBS for 10 and 30 minutes at 37°C. The cells were analyzed with the MACSQuant after two washes with PBS.

**Immunohistochemistry staining**

CAR T cells propagated following REM (22) as described above (see NBDG uptake assay) were stained with mitochondrial selective probe 100 nmol/L Mitotracker (Green; ThermoFisher Scientific) diluted in complete medium for 30 minutes at 37°C. Note that 1 × 10⁵ cells were embedded onto slides by cytospin, fixed with 4% PFA, and imaged using an LSM880 confocal microscope.

**Statistical analysis**

Analyses were performed using Prism (GraphPad Software, Inc.) or SAS 9.4. For normally distributed endpoints, raw or after logarithm transformation (e.g., flux), the Student t test was applied to comparisons between two independent, and one-way ANOVA models were applied to comparisons among 3 or more independent groups. The nonparametric Mann–Whitney test was applied to group comparisons for nonnormally distributed data. Linear mixed models were applied to repeated measures (e.g., flux) from the same subject to account for the variance–covariance structure. For survival data, the Kaplan–Meier method and Log-rank test were applied to survival function estimation and group comparisons. P values were corrected for multiple comparisons by the Holm method. P values of ≤0.05 were considered statistically significant.

**Results**

**ICV-administered CAR T cells exhibit potent antitumor activity against CNS lymphoma**

Administration of CD19-CAR T cells in all clinical settings to date has been via IV delivery. We tested the hypothesis that CD19-CAR T cells delivered by ICV infusion would result in superior CAR T-cell efficacy against CNS lymphoma. We first evaluated tumor growth and survival outcomes comparing IV and ICV delivery of CD19-CAR T cells to treat CNS lymphoma. To model CNS lymphoma (Fig. 1A), we implanted Daudi lymphoma cells engineered to express EGFP and fluic into the right forebrain of NSG mice. We treated mice 5 days after engraftment with either IV or ICV delivery of CD19-CAR T cells engineered using our clinical manufacturing platform in which Tn/mem cells are enriched prior to CAR transduction (25–27). When determining the CAR T-cell dose, we worked under the assumption that ICV-delivered CAR T cells, but not IV-delivered CAR T cells, would have direct access to CNS tumors (31), and achieving comparable therapeutic effects would require different cell doses. Therefore, in these experiments, we infused 3 × 10⁵ CAR T cells IV and 1.3 × 10⁶ CAR T cells ICV. We also treated tumor-bearing mice with mock-transduced T cells to control for potential xenoreactivity in our model. Tumors in mice receiving mock T cells delivered either IV or ICV progressed first in the brain and then systemically (Fig. 1B). By contrast, tumors in mice that received 1.3 × 10⁶ ICV-delivered CD19-CAR T cells were undetectable by bioluminescent imaging (BLI) by day 14, suggesting that ICV-delivered CD19-CAR T cells mediated rapid anti-CNS lymphoma activity. Although a high dose of IV-delivered CD19-CAR T cells (3 × 10⁵) also displayed antitumor activity, the kinetics of response were delayed; mice treated IV had significantly higher lymphoma burden at day 14 (P < 0.01) and did not have complete tumor eradication until day 21 (Fig. 1B).

To demonstrate that this observation was not limited to a specific tumor or CAR, we repeated our experiments using a different tumor and CAR model. B-cell activation factor receptor (BAFF-R) is commonly expressed on B-cell malignancies including MCL. We engrafted Jeko MCL cells engineered to express EGFP and fluc into the right forebrain of NSG mice and treated with second-generation BAFF-R CAR T cells (24) derived from Tn/mem cells are enriched prior to CAR transduction (25) and expressing mitochondrial read rate (e.g., flux) from the same subject to account for the variance–covariance structure. For survival data, the Kaplan–Meier method and Log-rank test were applied to survival function estimation and group comparisons. P values were corrected for multiple comparisons by the Holm method. P values of ≤0.05 were considered statistically significant.
ICV-administered CAR T cells completely eradicate both CNS and systemic lymphoma

Because we detected ICV-delivered CAR T cells in the periphery in two lymphoma/CAR models (Fig. 1D; Supplementary Fig. S2), we investigated whether ICV-administered CAR T cells could eliminate systemic lymphoma. We established a dual model of CNS and systemic lymphoma by engrafting Daudi lymphoma cells in the brain as described above and SC in the right flank of the same mouse (Fig. 2A). Although ICV-delivered mock-transduced T cells did not affect tumor growth compared with untreated controls, ICV-delivered CD19-CAR T cells induced significant regression of both CNS and systemic lymphoma ($P < 0.001$), with tumors at both sites undetectable by 21 days after infusion (Fig. 2B). Strikingly, mice treated with ICV-delivered CD19-CAR T cells remained tumor free for $>300$ days (Fig. 2B). In contrast, mice treated with an equal number of IV-delivered CD19-CAR T cells showed a significantly delayed response, with only 3 of 5 mice achieving tumor eradication at 43 days after CAR T-cell infusion. Moreover, all mice treated IV died before day 200 from relapse of both CNS and systemic lymphoma (Fig. 2B; $P < 0.01$), suggesting that IV-delivered CAR T cells failed to completely eradicate either form of lymphoma. Similarly, ICV delivery of CD19-CAR T cells modestly improved survival of mice bearing CNS and systemic Jeko.
tumors relative to ICV-delivered mock T cells (Supplementary Fig. S3A). However, there was no statistical difference in survival between IV- and ICV-delivered CD19-CAR T cells (P > 0.05), which is likely due to high systemic tumor burden in this model (Supplementary Fig. S3A). Together, these data indicate that ICV-delivered CAR T cells can migrate out of the CNS and have improved activity against systemic lymphoma when compared with IV-delivered CAR T cells.

ICV-administered CAR T cells exhibit memory phenotype and function with a broad T-cell receptor Vβ repertoire

To determine whether there were phenotypic differences between ICV- and IV-delivered CD19-CAR T cells after infusion that might explain the difference in potency, we analyzed CAR T cells isolated from peripheral blood of mice at the study endpoint by flow cytometry. Blood samples were collected at euthanasia, which occurred on day 334 for mice treated ICV and between days 79 and 180 for mice treated IV; the latter group were euthanized earlier due to tumor progression. Although mice treated ICV had undetectable tumor burden at the time of blood collection, we detected CAR T cells (CD3+; human CD45++; CAR+) in the blood (Fig. 2C and D). ICV-infused CAR T cells may exhibit a high degree of homeostatic proliferation and persistence, which are characteristics of memory T cells. When we compared the persisting CD19-CAR T cells, we found that ICV-delivered cells expressed significantly higher levels of CD28 compared with IV-delivered cells (P = 0.01), even following a longer in vivo engraftment (Fig. 2E), which may have contributed to disease elimination and CAR T-cell persistence. Consistent with the Daudi lymphoma model, CD19-CAR T cells were detected in peripheral blood of the Jeko MCL model 20 days after infusion. However, the trafficking and/or persistence in this model appears to be dependent on the presence of tumor as CD19-CAR T cells were detected only if there was concurrent brain or systemic lymphoma (Supplementary Fig. S3B).

To understand whether antilymphoma activity was due to clonal or polyclonal CAR T-cell expansion, human T cells harvested at the study endpoint were restimulated for 14 days using REM, followed by T-cell receptor (TCR) repertoire analysis. The preinfusion product, and both the ICV- and IV-delivered CAR T cells all displayed broad TCR Vβ usage (Fig. 2F; Supplementary Fig. S4), indicating that the CAR T cells mediating antilymphoma activity were polyclonal.

Antigen-primed CAR T cells potently eradicate systemic lymphoma and resist tumor rechallenge

To explain the observation that ICV-infused CAR T cells more effectively eradicated both CNS and systemic lymphoma in our dual lymphoma model (Fig. 2), we hypothesized that ICV-infused CAR T cells may have been primed by exposure to tumor antigen in the CNS, resulting in potent activity against systemic lymphoma. To assess the contribution of antigen priming on the potency of ICV-delivered CAR T cells, we designed an in vitro priming model in which CAR T cells were cocultured with irradiated tumor cells for 1 hour before ICV injection into mice bearing only systemic lymphoma. Mice treated with in vitro–primed CD19-CAR T cells delivered ICV had significant regression of systemic lymphoma when compared with mice treated with unprimed CD19-CAR T cells by day 28 (P < 0.001), leading to a significant survival advantage (P < 0.01; Fig. 3A) and supporting the hypothesis that priming may further improve antitumor activity and persistence of ICV-delivered CAR T cells. When surviving mice were subsequently rechallenged with new systemic lymphoma 133 days after CAR T-cell infusion, primed and unprimed CAR T cells delivered ICV were equally efficient at rejecting systemic lymphoma (Fig. 3A; Supplementary Fig. S5A).

To determine if the enhanced CAR T-cell function we observed following antigen priming was independent of exposure to CSF, we treated mice bearing systemic lymphoma with primed or unprimed CD19-CAR T cells IV. Consistent with previous observations, primed CAR T cells delivered IV induced superior tumor regression when compared with unprimed CAR T cells (P < 0.001) on day 28 (Fig. 3B). All mice that received unprimed CD19-CAR T cells IV died before day 113. In contrast, 3 of 5 mice that received primed CAR T cells IV survived >135 days, and all surviving mice resisted tumor rechallenge (Fig. 3B; Supplementary Fig. S5B). When we examined the peripheral blood of mice 133 days after CAR T-cell infusion, we observed significantly higher levels of CAR T cells in mice treated with either unprimed ICV-delivered or primed IV-delivered CAR T cells (Fig. 3C; P < 0.001). Overall, these data support the notion that T-cell activation of ICV-delivered CAR T cells by exposure to lymphoma in the brain (i.e., priming) does contribute in part to enhanced CAR T-cell persistence and potency against systemic lymphoma.

ICV-delivered CAR T cells show superior proliferative potential compared with IV-delivered CAR T cells

Because antigen priming played a role in the potency of both IV- and ICV-delivered CAR T cells against systemic lymphoma, we next assessed whether CAR T cells would traffic to systemic tumors and proliferate in the absence of priming. To track CAR T cells in vivo, CD19-CAR T cells were transduced to express EGFP and fluo (Fig. 4A; Supplementary Fig. S6A) and administered ICV or IV into mice bearing systemic lymphoma. Both IV- and IV-delivered CAR T cells migrated to and efficiently infiltrated the systemic tumors as assessed by BLI (Fig. 4B). Interestingly, the bioluminescent signals were significantly higher in ICV- versus IV-treated mice at day 35 (P < 0.001; Fig. 4C), indicating greater in vivo expansion of ICV-delivered CAR T cells. This expansion was dependent on engagement of the CAR as mock T cells did not expand in vivo (Supplementary Fig. S6B) and tumor volumes were not controlled by mock T cells (Supplementary Fig. S6C). When we examined CAR T-cell persistence, we observed that the ratio of CAR+ to CAR− human CD45+ cells increased over time for both IV- and IV-treated groups (Fig. 4D and E). Thus, although both ICV- and IV-delivered CAR T cells can migrate to systemic lymphoma, ICV-delivered cells have superior capacity for proliferation and persistence.

CSF conditions CAR T cells for enhanced memory and reduced exhaustion

Exposure to CSF may enhance CAR T-cell potency

We observed that both IV- and IV-delivered CAR T cells have similar homing potential to systemic tumors even in the absence of antigen priming (Fig. 4). We therefore explored why ICV delivery yielded CAR T cells possessing superior antilymphoma capacity. We hypothesized that exposure to CSF upon ICV administration may modify CAR T cells to enhance their function. Glucose and potassium, which are known to be involved in T-cell memory and function (32–36), are found at lower levels in CSF (60 mg/dL of glucose; 2.8 mEq/L of potassium) compared with serum (90 mg/dL of glucose; 4.5 mEq/L of potassium; ref. 37). To investigate if exposure to CSF affects CAR T-cell gene expression, we cultured CD19-CAR T cells in regular RPMI (Invitrogen; 200 mg/dL of glucose; 5.3 mEq/L of potassium) or CSF.
from healthy human donors for 48 hours and analyzed for expression of genes associated with memory, effector function, and metabolism. Across 4 different donors, we consistently observed elevated levels (>1.3-fold change) of genes related to survival and memory function, including BCL2, BCL2L11, KL2, and IL7Rα (CD127), and decreased expression (>1.3-fold change) of effector differentiation genes like PRF1, IFNγ, GZMB, and TBX21 in CAR T cells cultured in CSF when compared with RPMI (Fig. 5A). Of note, numerous other genes regulating glucose metabolism, including CPT1A and Pgam-1 (33), were downregulated in CSF cultures. These data support the hypothesis that CAR T cells cultured in CSF underwent metabolic reprogramming to favor memory formation. Consistent with the transcriptional profile suggesting that CSF maintains CAR T cells in a less differentiated state compared with RPMI (Fig. 5A), CAR T cells cultured in CSF produced significantly lower levels of effector cell–related Th1 and T-cell–inhibitory Th2

**Figure 3.**
Antigen-primed CAR T cells possess better effector memory function and can resist tumor rechallenge. NSG mice were inoculated with SC injections of 3 × 10⁶ Daudi lymphoma cells in the right flank 19 days prior to CAR T-cell treatment. On the day of CAR T-cell infusion, 2 × 10⁶ CD19-CAR T cells were primed with irradiated Daudi cells at 10:1 (CAR:tumor) ratio for 1 hour prior to infusion by ICV (A) or IV (B) delivery. Mock T cells were infused into control mice. Flux (photons/sec) was determined by measuring bioluminescence by live imaging once per week. Note that 135 days after CAR T-cell infusion, surviving mice were rechallenged (dotted lines) with Daudi lymphoma cells by SC injection. Naive mice were inoculated with Daudi lymphoma cells at rechallenge as controls. Mean ± SDs from 5 mice per group are presented. Linear mixed models were used to compare flux (logarithm transformed) among different groups over time, and log-rank test was used to compare survival functions among the groups. If a mouse died during imaging processes with no prior sign of tumor progression, then it was considered an anesthesia-related death, and the mouse was excluded from survival analysis. *, P < 0.05; **, P < 0.01; and ***, P < 0.001. Blood was collected retro-orbitally 133 days after CAR T-cell treatment and analyzed for expression of human CD45, CD3, and CAR (EGFR) by flow cytometry. No mice survived in the unprimed CD19-CAR IV group. Significance was determined by one-way ANOVA test. ***, P < 0.001.
ICV-infused CAR T cells exhibit similar trafficking but superior proliferation and persistence potential when compared with IV-delivered CAR T cells. A, CD19-CAR T cells were transduced with EGFP-fluc and expanded in vitro. NSG mice were implanted SC with Daudi lymphoma in the right flank. Nineteen days after tumor engraftment, 2 x 10^6 EGFP+ Fluc+ CAR T cells were administered ICV or IV, and CAR T-cell proliferation was determined by measuring bioluminescence by live imaging every other day (B–C). The same scale was used for each time point. D–E, Blood was collected at different time points after CAR T-cell infusion, and T-cell (human CD45+) and CAR+ T-cell (EGFR+) levels were detected by flow cytometry. Mean ± SDs from 5 mice per group are presented. Significance was determined with the Mann-Whitney test; *, P < 0.05 and ***, P < 0.001. Experiments were repeated twice, and representative data are presented.
cytokines upon in vitro stimulation with Daudi lymphoma cells compared with RPMI-cultured CAR T cells (Fig. 5B).

To control for differences between CSF and regular RPMI, we performed experiments with modified RPMI medium that mimics the levels of glucose and potassium in CSF (60 mg/dL glucose; 2.8 mEq/L potassium). CD19-CAR T cells generated in modified RPMI showed lower fold expansion than cells generated in regular RPMI (Supplementary Fig. S7A), which may be a mechanism that prevents differentiation and maintains memory. In support of this, CD19-CAR T cells expanded in modified RPMI expressed elevated levels of memory marker CD28 compared with cells generated in regular RPMI (P < 0.001; Fig. 5C) and activation marker CD25 (P < 0.01), but did not show change in the exhaustion markers LAG3, Tim3, and KLRG (Supplementary Fig. S7B and S7C). Remarkably, these cells sustained significantly higher levels of CD28 when compared with cells cultured in regular RPMI even after cultures were switched from modified to regular RPMI.
regular RPMI medium for 14 days (Fig. 5C). Moreover, these cells maintained effector capabilities, as indicated by comparable CD107a expression (i.e., degranulation) upon coculture with Daudi target cells (Fig. 5D). Overall, our data suggest that the improved function of ICV-delivered CAR T cells may be attributed to reprogramming to memory T cells in the CSF environment.

Distinct gene signatures of in vivo persisting ICV and IV CAR T cells

To quantitatively dissect differences in expression of memory, differentiation, and immune checkpoint markers, we harvested CAR T cells delivered ICV or IV from mice bearing both CNS and systemic lymphoma and performed single-cell RNA-seq. Human T cells were isolated from mouse bone marrow 68 days after CAR T-cell infusion. Clustering analysis identified 5 clusters; t-SNE plots demonstrated that CAR T cells harvested from ICV–treated mice are enriched in cluster 3, whereas CAR T cells from IV–treated mice are enriched in cluster 1 (Fig. 6A). In agreement with in vitro CSF–treated cells (Fig. 5), in vivo persisting ICV–delivered CAR T-cell–enriched cluster 3 is characterized by higher levels of hallmark memory genes (KLF2, BCL2L11, BCL2, IL7R, CD27, and CD28) and lower levels of differentiation and effector genes (PDCD1, EOMES, FOX, TBX21, IFNG, PRF1, GZMH, and GZMB) when compared with IV–delivered CAR T-cell–enriched cluster 1 (Fig. 6B). Overall gene expression patterns, as shown in the

Figure 6.
ICV- and IV-delivered CAR T cells that persist in vivo have distinct gene signatures. T cells were harvested from the bone marrow of mice 68 days after ICV or IV CAR T-cell infusion, and the samples from 1 mouse in each group were subjected to single-cell RNA-seq (scRNA-seq) analysis. The subsequent analysis of human gene data was performed using “Seurat” package v3.0 and R scripts. t-SNE visualization plot of scRNA-seq data (A), and dot plots and feature plots in the identified cell clusters (B) were generated for specific T-cell populations. ICV-delivered cells were enriched in cluster 3, whereas IV–delivered cells were enriched in cluster 1. C, Cluster-specific markers were identified to generate a heatmap.
heatmap in Fig. 6C, display distinct gene signatures between the clusters enriched for ICV- and IV-delivered CAR T cells.

**Enhanced memory and reduced exhaustion of ICV-delivered CAR T cells**

To investigate if the gene expression patterns that distinguished ICV- from IV-delivered CAR T cells (Fig. 6) resulted in enhanced memory and/or reduced exhaustion, we performed an in vitro serial restimulation with CAR T cells harvested from the spleens of mice at the study endpoint. T cells were stimulated with REM for 2 cycles (14 days/cycle), which included both OKT3- and CD19-mediated antigen stimulation (Fig. 7A). CAR T cells from ICV-treated mice expanded 9.1 ± 0.9-fold from REM1 to REM2, whereas CAR T cells from IV-treated mice had either lower expansion or reduction in overall numbers (1.7 ± 1.6-fold; Fig. 7B), suggesting that ICV-delivered CAR T cells resisted T-cell activation–induced cell death (38, 39) and had superior expansion potential. Despite 334 days of in vivo incubation and repeated in vitro stimulation, CAR T cells from ICV-treated mice maintained higher levels of CD127, CD62L, and CD161—markers that represent central memory T cells and...
Low glycolytic status of the ICV- and IV-delivered CAR T cells harvested from mice, we examined their capacity for uptake of the glucose analogue NBDG. We observed that CD62L+ cells, which are enriched in those isolated from ICV-treated mice (Fig. 7C), exhibited significantly lower (P < 0.01) NBDG uptake than CD62L− cells (Fig. 7D and E), supporting the memory-like behavior of these CD62L+ cells (33, 41). To meet differing energy requirements, effector T cells activate mitochondrial fission and subsequent fragmentation, whereas memory T cells inhibit mitochondrial fission, leading to mitochondrial elongation (42, 43). In line with these reported observations, T cells from ICV-treated mice maintained fused mitochondrial networks that appeared elongated, whereas T cells from IV-treated mice had punctate mitochondria (Fig. 7F).

Discussion

Although CAR T-cell therapy has shown great promise in the treatment of hematologic diseases, the generation of durable responses against tumor relapse remains an obstacle. We here propose that ICV delivery of CD19-CAR T cells holds superior potential to treat CNS and systemic lymphoma, which supports the conclusions of two recently published studies that demonstrate that ICV-delivered CAR T cells more effectively control primary and metastatic brain tumor cells than IV delivery (41, 44). In the present study, ICV-delivered CAR T cells potently eradicated both CNS and systemic lymphoma, leading to lymphoma elimination, in conditions where equal or higher doses of IV-delivered CAR T cells did not yield disease elimination (Figs. 1 and 2). Furthermore, CAR T cells isolated from ICV-treated mice showed increased phenotypic and functional memory characteristics (Figs. 2, 6, and 7), which we were able to recapitulate by in vitro culture with CSF (Fig. 5). These findings suggest that CAR T cells are conditioned by exposure to CSF in the ICV environment to promote antitumor activity and memory formation.

Importantly, this study examined how persistence of CAR T cells is affected by route of administration. CAR T cells delivered ICV were detectable in peripheral blood of mice >300 days after infusion, even without detectable lymphoma (Fig. 2). We acknowledge that the possibility of xenoreactivity of CAR T cells is a potential limitation of our model. However, our studies were well controlled with equal dosing of nontransduced (mock) T cells, which demonstrated no antilymphoma activity. Moreover, we followed the duration of response to ICV-delivered CAR T cells for over 11 months (334 days) and did not observe any evidence of toxicity due to graft-versus-host disease (GVHD), which would indicate xenoreactivity. Absence of GVHD suggests that there is limited engagement of the endogenous TCR in our model and, therefore, xenoreactivity likely does not affect our conclusions. In line with these observations, a recent study by Mulazzani and colleagues (45) showed that intracerebroventricular infusion of CAR T cells exhibits superior antitumor efficacy and persistence compared with IV delivery, where xenoreactivity of CAR T cells is ruled out using mock T cells. These data support our observation that differences in outcomes in mice treated with ICV and IV delivery reflected differences in capacity for antitumor activity promoted by the two routes of delivery. The long-term persistence of memory CAR T cells following ICV administration in absence of antigen, also observed by Mulazzani and colleagues (45), could be due to homeostatic mechanisms driven by factors such as cytokines that are species cross-reactive, such as IL7 (46, 47).

ICV-delivered CAR T cells exhibited a higher degree of memory phenotype than did IV-delivered CAR T cells (Figs. 6 and 7), and an elongated mitochondrial morphology consistent with maintaining memory T-cell persistence (Fig. 7; ref. 43). With t-SNE clustering and underlying data output, single-cell RNA-seq datasets revealed distinct gene signatures between ICV- and IV-delivered CAR T cells that supported evolving memory features of ICV-delivered CAR T cells but advanced differentiation signatures of IV-delivered CAR T cells (Fig. 6).

We propose that exposure of CAR T cells to CSF within the ICV environment leads to a metabolic reprogramming that favors the formation of memory. Proper engagement of metabolic pathways is critical to fulfilling the nutrient demands of immune cells (33, 35, 36, 48–50), and inhibition of glycolysis enhances memory T-cell formation (33, 36). However, elevated extracellular potassium is required to support effective function through the PPP2A pathways (51). Compared with serum, CSF contains lower levels of glucose and potassium, and CAR T cells cultured in CSF or modified RPMI compared with RPMI with glucose and potassium levels similar to serum had changes in gene expression that promoted a memory phenotype (Fig. 5).

In keeping with clinical observations that reactivating CAR T cells with antigen further augments their efficacy (7), our data showed that antigen-primed CAR T cells possessed significantly enhanced antitumor activity, expansion, and persistence regardless of route of administration when compared with unprimed CAR T cells (Fig. 3). These data indicate that antigen priming of CAR T cells contributes to improved antitumor activity, independent of the CSF microenvironment.

Collectively, the data presented here provide rationale to clinically evaluate ICV delivery of CAR T cells to treat primary CNS lymphoma and systemic lymphoma with CNS involvement, as we demonstrated that a single dose of ICV-delivered CAR T cells has the potential to cure both primary CNS and systemic lymphoma in mice. Unlike classical T-cell differentiation patterns, where enhanced effector function is associated with impaired memory and persistence, ICV-infused CAR T cells successfully acquired complete effector function for antitumor activity while maintaining memory function for long-term immune surveillance and resistance to tumor rechallenge. These differences in potency and longevity should be evaluated in other systemic tumor types where CNS involvement is common and may represent a generalizable strategy to improve CAR T-cell therapy.
ICV-Delivered CD19-CAR T Cells for CNS and Systemic Lymphoma

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References


The Cerebroventricular Environment Modifies CAR T Cells for Potent Activity against Both Central Nervous System and Systemic Lymphoma

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