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# Supplementary Information for

## Long-term *in vivo* microscopy of CAR T cell dynamics during eradication of CNS lymphoma in mice

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Supplementary text
Figs. S1 to S10
Captions for movies S1 to S8

**Other supplementary materials for this manuscript include the following:**

Movies S1 to S8



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## Supplementary Methods

### Perioperative care

For cranial window implantation and stereotactic tumor cell injection, anesthesia was induced via intraperitoneal injection of a mixture of medethomidine, midazolam, and fentanyl. Only for window implantation, anesthesia was followed by s.c. injection of dexamethasone. For window implantation and stereotactic injections, cefotaxime was injected after induction of anesthesia. 30 minutes before blood sampling, mice were s.c. injected with buprenorphine. Anesthesia was induced with isoflurane in oxygen. After surgeries, mice were treated with buprenorphine and evaluated for development of symptoms at least three times per week using a standardized test for neurological symptoms considering body weight, spontaneous movement, reaction to blunt touch, and balance testing. Mice were euthanized if symptoms of predefined severity occurred or weight loss exceeded more than 20%.

### Two photon laser scanning microscopy

Signals were collected with three non-descanned PMTs (G6780-20, Hamamatsu). The following filter combinations were used: second harmonic generation (SHG) signals were collected with reflection of two long-pass dichroic mirrors (560 nm and 495 nm). eGFP signal was collected with the same long-pass dichroic mirrors (560 nm and 495 nm) and a band-pass filter (525/50). TdTomato signal was collected with two long-pass dichroic mirrors (560 nm and 615 nm) and a band-pass filter (593/40). For FITC-dextran and tdTomato, excitation wavelength was 775 nm. For eGFP, tdTomato and SHG signal, an excitation wavelength of 920 nm was used. z-stacks acquired had dimensions of either 450 × 450 or 555 × 555 µm (as indicated) with 400 µm depth, a z-interval of 5 µm and a resolution of 1024 × 1024 pixels or 512 × 512 pixels. Time lapse movies were acquired at dimensions of 450 × 450 or 555 × 555 µm with 65 µm depth, a z-interval of 5 µm at a resolution of 1024 × 1024 pixels acquired every 30 seconds. Imspector (LaVision Biotec) software was used for image acquisition.

### Image analysis

For mosaic creation, maximum intensity projections of three-dimensional stacks were created using the Z-Project plugin of ImageJ/Fiji, followed by manual alignment using the MosaicJ function. For noise reduction, the despeckle function (ImageJ/Fiji. Median filter: each pixel is replaced by the median intensity value in its 3 × 3 neighborhood) was used. Quantification and analysis of z-position and cell velocity of intratumoral and intracerebral CAR T cells were done using Imaris.

### Immunofluorescence

For immunofluorescence microscopy, the following primary antibodies were used: goat anti-human CD20 (ab194970, abcam), rat anti-mouse CD3 (MAP4841, R&D Systems). The following secondary antibody conjugates were used: donkey anti-goat Alexa Fluor (AF) 594 (A11D58, Thermo Fisher Scientific), donkey anti-rat AF594 (A21203, Thermo Fisher Scientific), donkey anti-rat AF488 (A21208, Thermo Fisher Scientific). For chip cytometry, the following antibodies were used: PE-conjugated anti-CD11c (Biolegend, clone N418), PE-conjugated CD27 (LifeTech, clone LG7.F9), AF488 (Biolegend, clone 17A2).

**Detailed author contributions**

MM designed, established and performed the experiments, analyzed the data, prepared most of the figures and wrote the manuscript. SF established the CAR T cell transduction and cloned the novel CAR construct. SF, IMH, SL, XZ, HIA, JL, WZ, SD, MS, MR contributed to the experimental work, part of the data analysis (SF, IMH, SL) and prepared two figures (SF). VB and DB contributed to the design of the experiments, provided methodological input and the infrastructure for the generation and handling of CAR T cells. MBB, MD and AS provided clinical as well as methodological input and provided lab infrastructure (AS). LB had the idea to establish the animal model, designed and coordinated the research project, co-wrote the manuscript and supervised the project. All authors have read, revised and approved the manuscript.

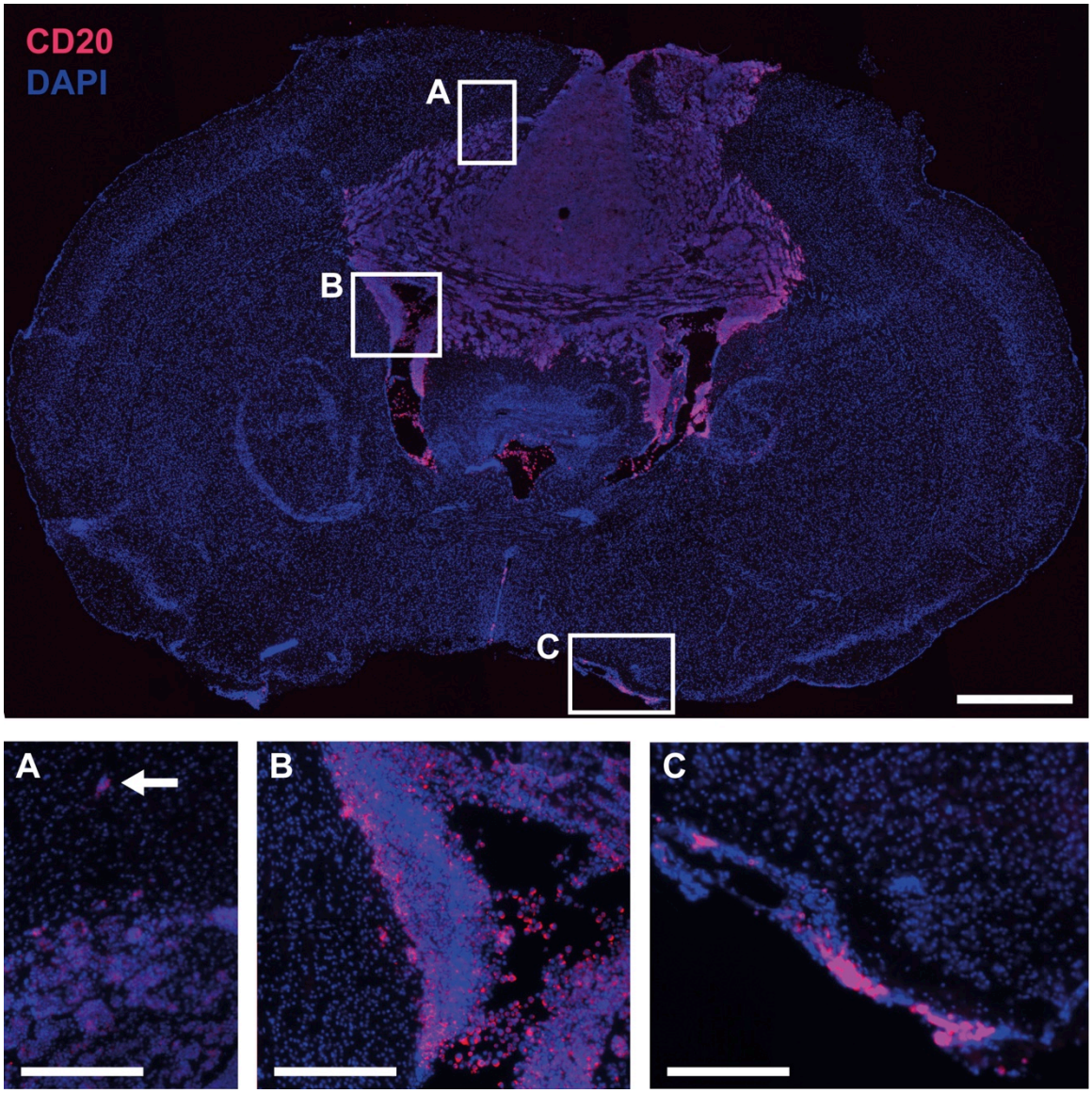


**Fig. S1. Mouse model of orthotopic PCNSL growth.** Representative immunofluorescence staining of a mouse brain 35 days after implantation of U2932 cells. Experimental PCNSL exhibits an intracerebral (**A**) growth pattern with intra- / periventricular (**B**), meningeal and perivascular (**C**) tumor cells. Small clusters of infiltrating tumor cells distant from the solid tumor can be seen (**A**, arrow). Sections were stained with DAPI (blue) and anti-human-CD20 antibodies (pink, tumor cells). Scale bars, 1 mm (top) or 250 µm (below). Representative immunofluorescence staining of 17 mice from 5 experiments.

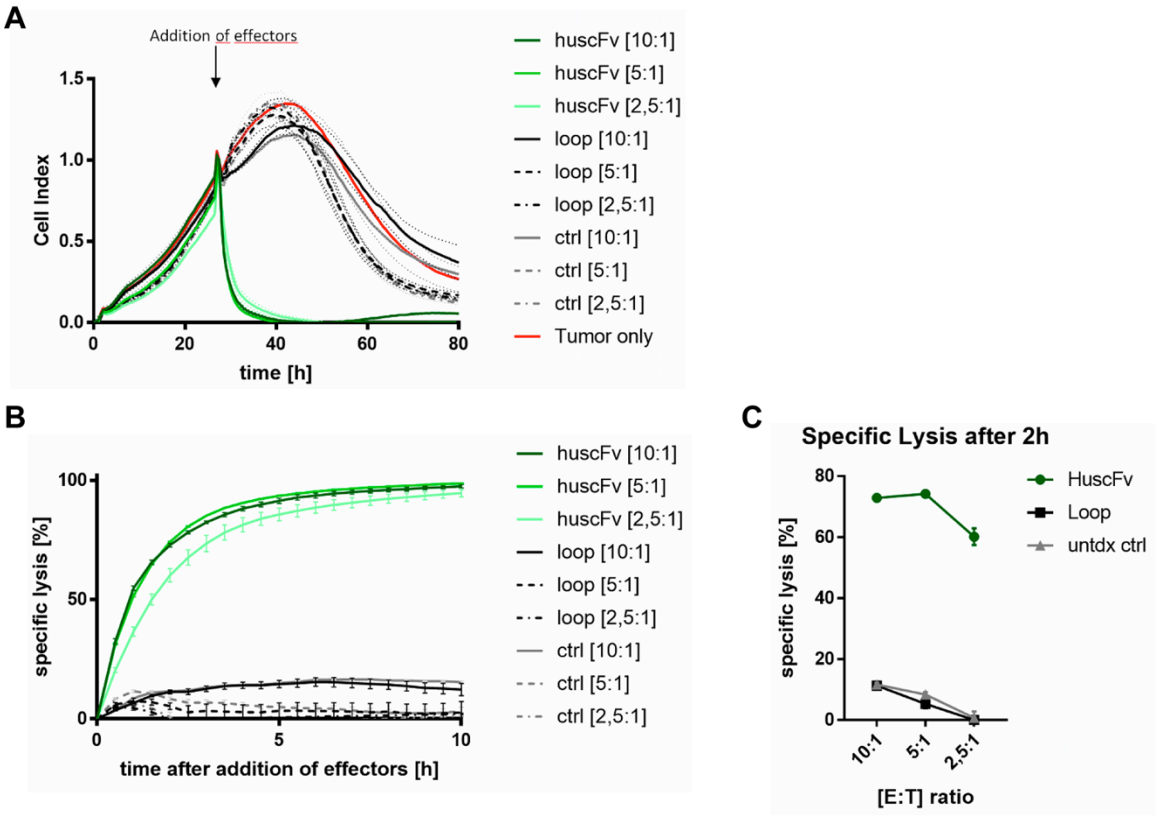


**Fig. S2. h19m28z CAR T cells exhibit CD19-dependent cytotoxic effects independent of bystander cells.** Specific lysis was measured via xCELLigence RTCA impedance monitoring for 24 h, using adherent hCD19$^{+}$HEK293 target cells and h19m28z CAR (green), mock CAR (black) or untransduced control (gray) T cells in a 10:1, 5:1 and 2,5:1 (E:T) ratio. Specific target cell lysis was only observed with h19m28z CAR T cells in a dose dependent manner. Data are shown as mean +/- s.e.m. and are representative of 3 independent experiments performed in triplicate.

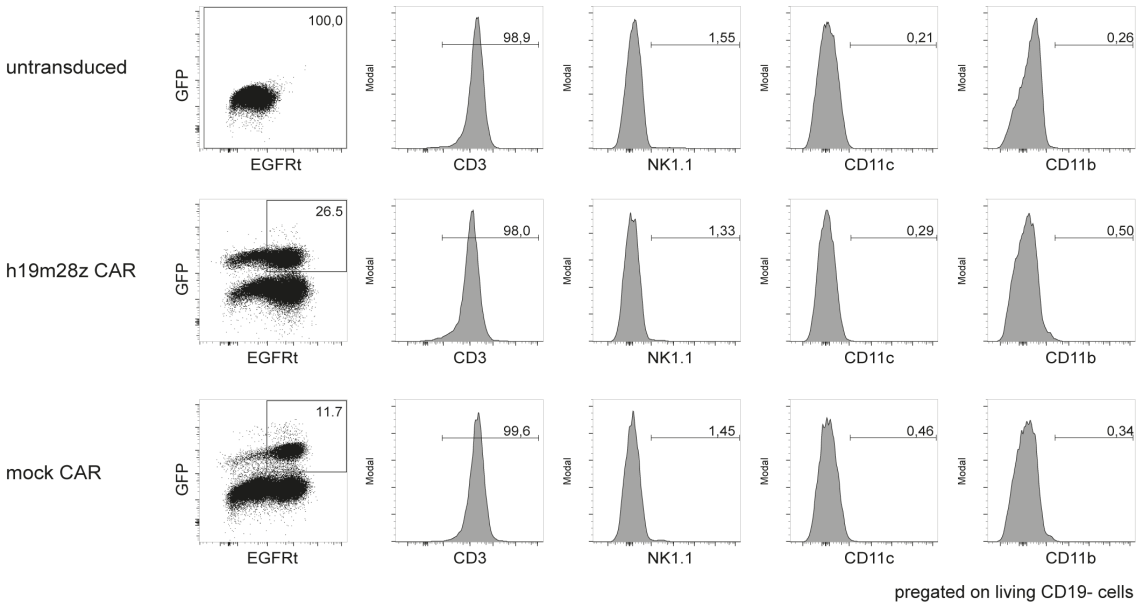


**Fig. S3. FACS analysis illustrates that after cell culture, stimulation and transduction, ≥ 98% of sorted cells are CD3$^+$ T cells, while NK cells amount to ≤ 1.5%.** Representative FACS plots of 3 replicates from one experiment.

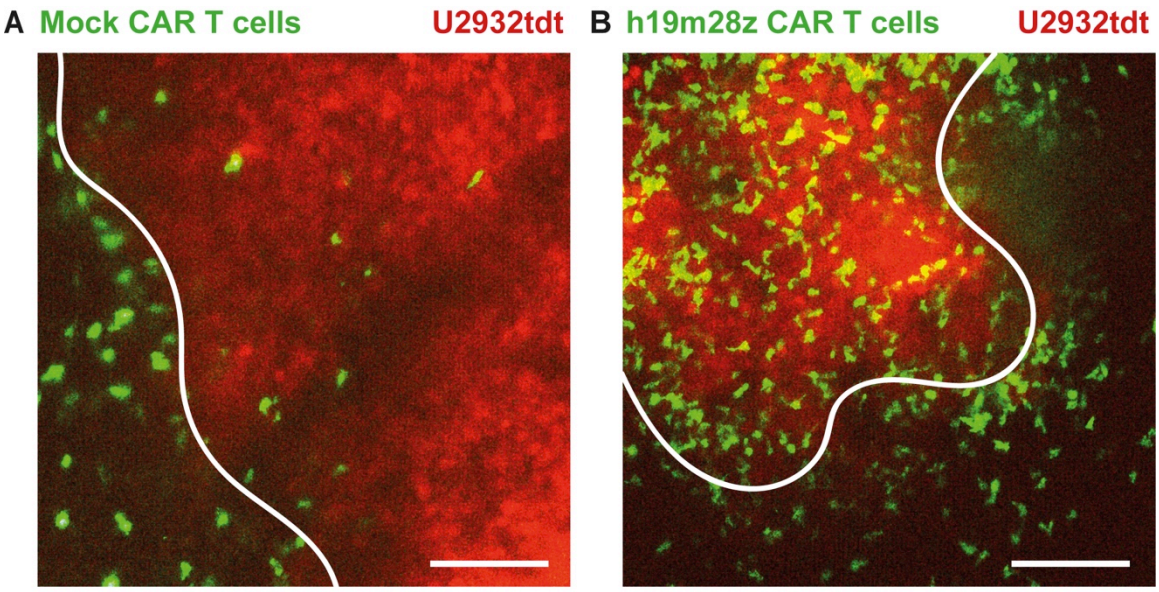


**Fig. S4. Mock CAR T cells accumulate at the tumor margin, whereas h19m28z CAR T cells accumulate intratumorally at high numbers.** MIPs of 450 x 450 x 55 µm (x/y/z), 100 µm below the most superficial tumor portion. U2932tdt cells in red, mock (**A**) or h2938z (**B**) CAR T cells in green. Scale bars, 100 µm. Representative MIPs of 4 mice per group from 2 independent experiments.

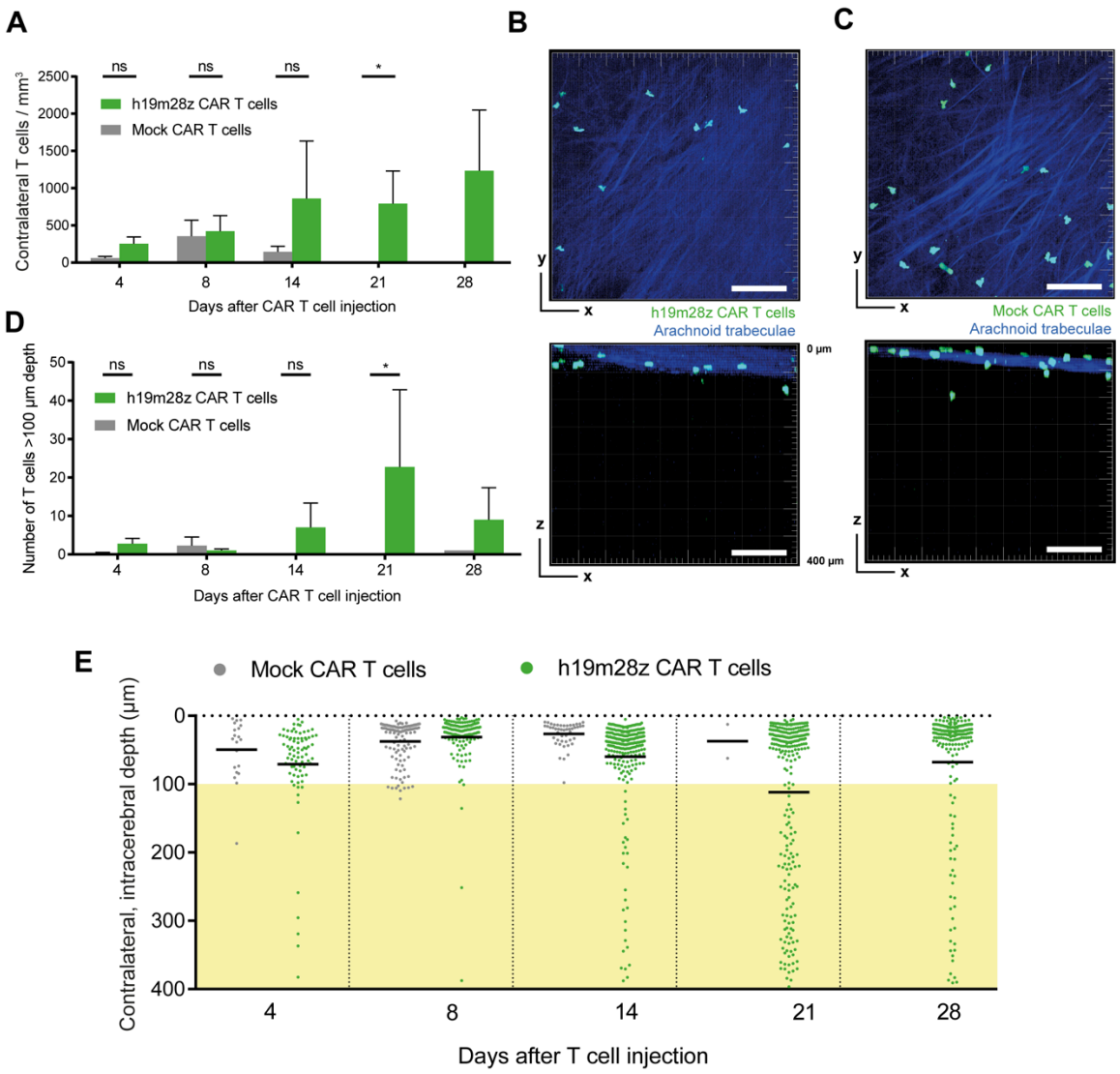


**Fig. S5. Contralateral, intracerebral h19m28z CAR T cells are present in higher numbers and persist longer than mock CAR T cells.** (**A**) Extratumoral CAR T cell numbers in mice treated with either h19m28z (green) or mock CAR T cells (gray, three-dimensional quantification of the corresponding ROI in the hemisphere contralateral to tumor injection site of 4 mice per group per time point). (**B** and **C**) Representative MIPs in axial (xy, upper panels) and sagittal (xz, lower panels) orientation illustrating extratumoral h19m28z (B) and mock (C) CAR T cells within the contralateral brain parenchyma 14 days after CAR T cell injection. Second harmonic generation signal of arachnoid trabeculae (collagen, blue). Scale bars, 100 µm. (**D**) Number of contralateral, intraparenchymal h19m28z (green) or mock (gray) CAR T cells > 100 µm beneath the most superficial part of arachnoid mater. (**E**) Distance of meningeal (<100 µm) and intraparenchymal (>100 µm) CAR T cells from brain surface (n = the ROI contralateral of tumor injection of 4 mice per group from 2 independent experiments. Each point represents one mock (gray) or h19m28z CAR T cell (green). After tumor overgrowth of the ROI contralateral to tumor injection (day 28: 0 of 4 mice in the h19m28z group, 3 of 4 in the mock group), T cell number and position have been excluded. Data are shown as mean + s.e.m. (A, B) or mean (E). Mann-Whitney *U* test (A, B, E). ns = not significant.

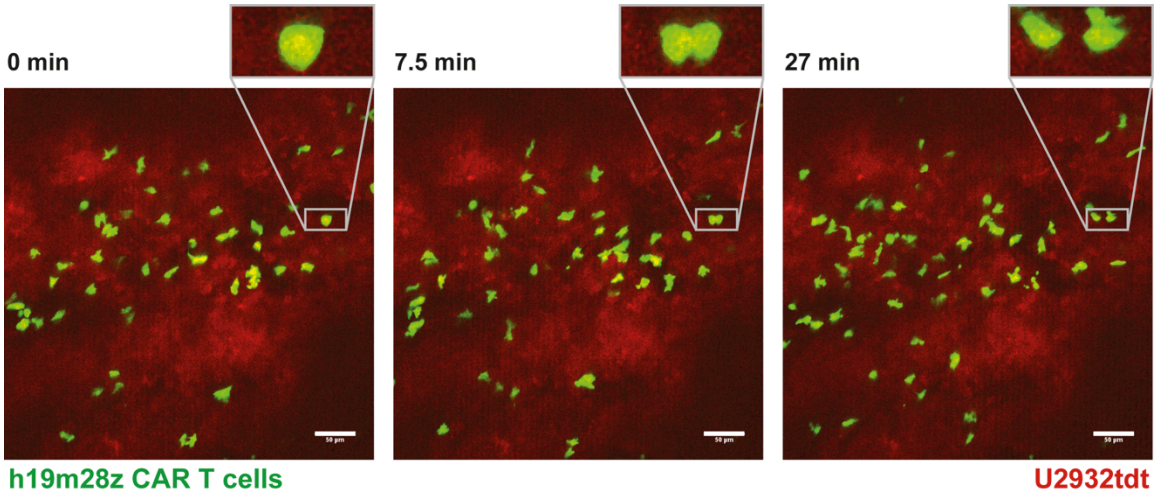


**Fig. S6**. **Intratumoral h19m28z CAR T cell mitosis.** TPLSM time lapse movie of 30 minutes duration (Movie S4). MIPs of 450 x 450 x 66 µm (x/y/z), image acquisition every 30 seconds. Representative data of 4 mice from 2 independent experiments. Scale bars, 50 µm.

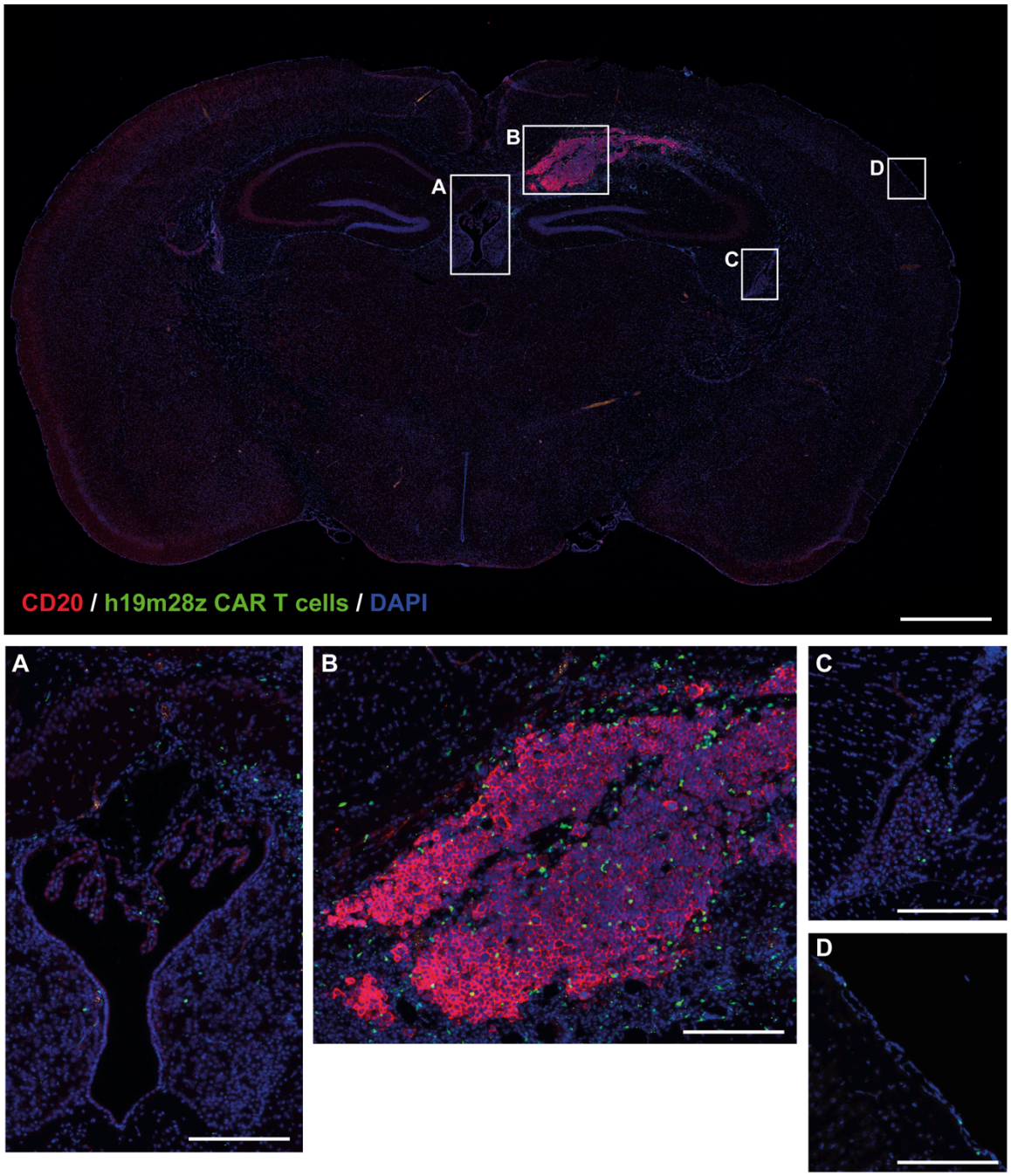


**Fig. S7. h19m28z CAR T cell treatment leads to high intratumoral, periventricular and meningeal CAR T cell numbers.** Representative mouse brain 28 days after h19m28z CAR T cell injection (42 days after tumor injection). Note the high numbers of intratumoral (**B**), intraventricular (**A** and **C**), subependymal (**A** and **C**), and meningeal (**D**) CAR T cells. Immunofluorescence staining of U2932tdt cells with CD20 immunostaining (red), eGFP signal of h19m28z CAR T cells (green) and DAPI nuclear staining (blue). Scale bars, 1 mm (big panel) or 200 µm (A, B, C, D). Representative immunofluorescence of 7 mice from 2 independent experiments.

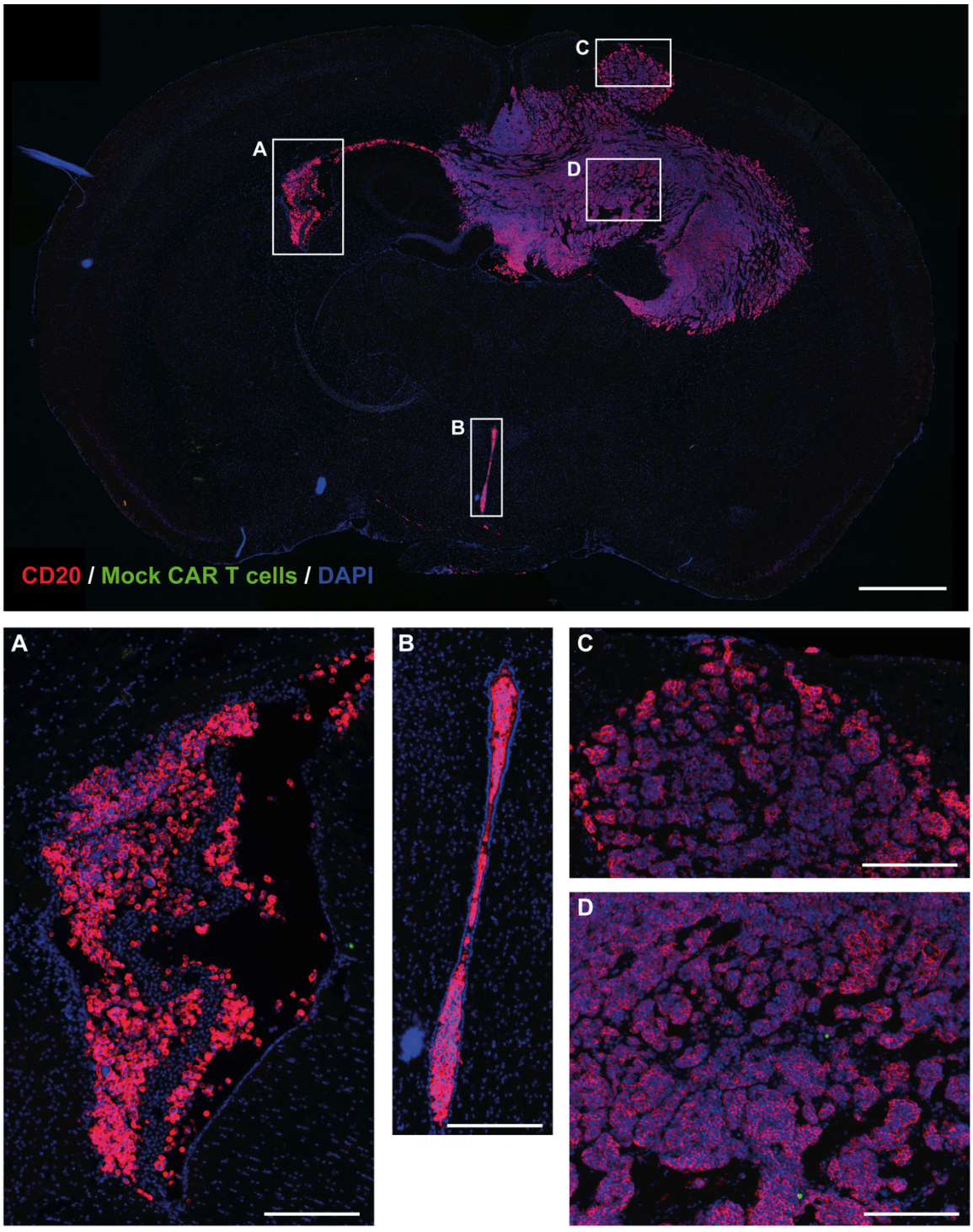


**Fig. S8. Mock CAR T cell treatment leads to growth of a large tumor and low intratumoral and intracerebral CAR T cell numbers.** Representative mouse brain 28 days after mock CAR T cell injection (42 days after tumor injection). Note the intraventricular (**A** and **B**) and intraparenchymal (**C** and **D**) tumor growth with low numbers of mock CAR T cells. Immunofluorescence staining of U2932tdt cells with CD20 immunostaining (red), eGFP signal of mock CAR T cells (green) and DAPI nuclear staining (blue). Scale bars, 1 mm (big panel) or 200 µm (A, B, C, D). Representative immunofluorescence of 7 mice from 2 independent experiments.

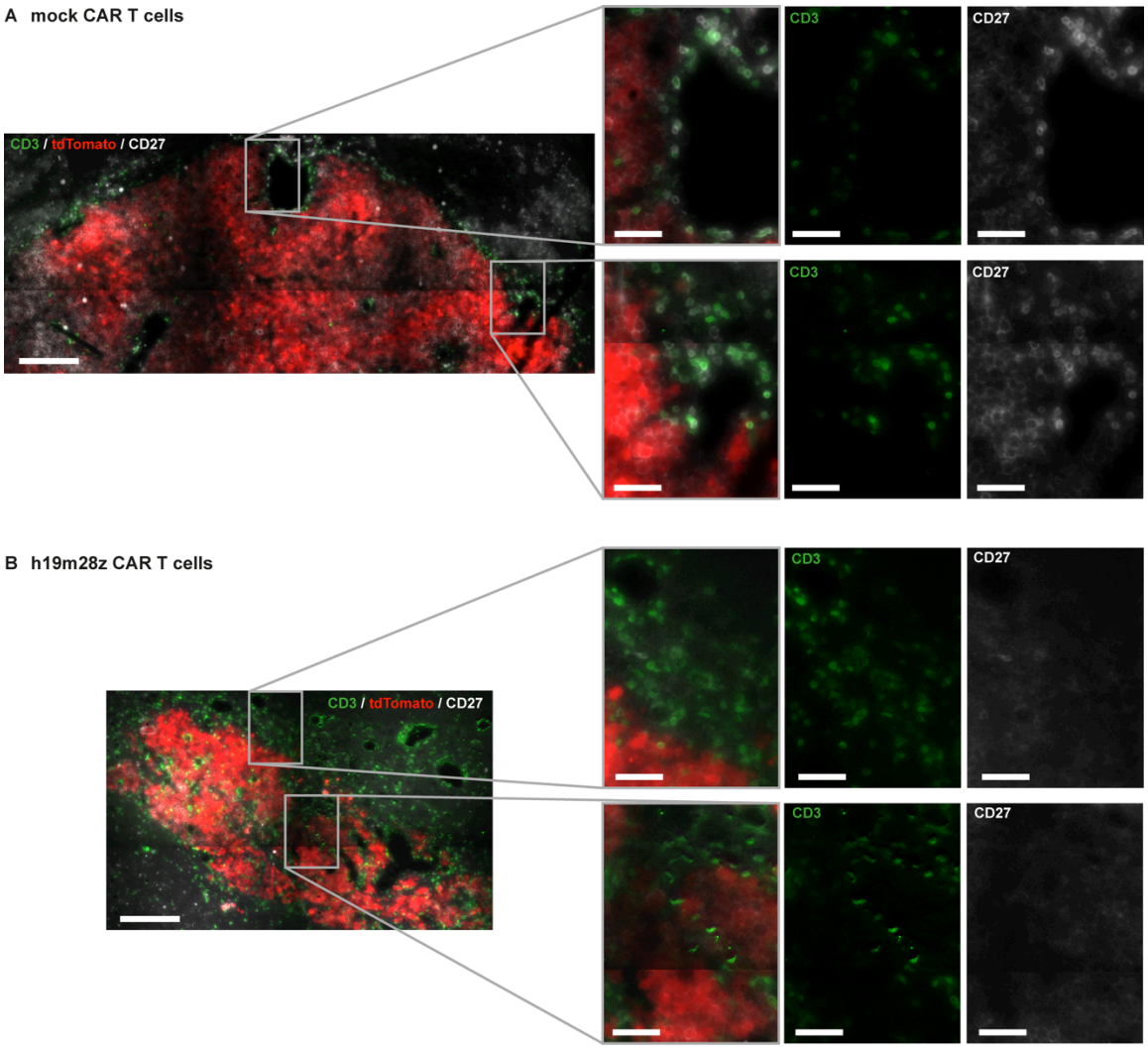


**Fig. S9. Mock CAR T cells abundantly express CD27, whereas h19m28z CAR T cells exhibit virtually no CD27 expression.** (**A**) Mock CAR T cells (green) surrounding intracerebral lymphoma (red) are $CD27^+$ (gray), indicating no relevant differentiation into effector T cells. (**B**) Peritumoral h19m28z CAR T cells however contain only few $CD27^+$ cells, suggesting CD27 downregulation, as is also observed in effector T cells. Immunofluorescence showing U2932 cells (tdTomato, red) and $CD27^+$ cells (gray) 28 days after extratumoral, intracerebral mock CAR T cell injection (CD3 staining, green). Scale bars, 200 µm (left) and 25 µm (inlays, right). A representative sample of two mice per group of two independent experiments is presented.

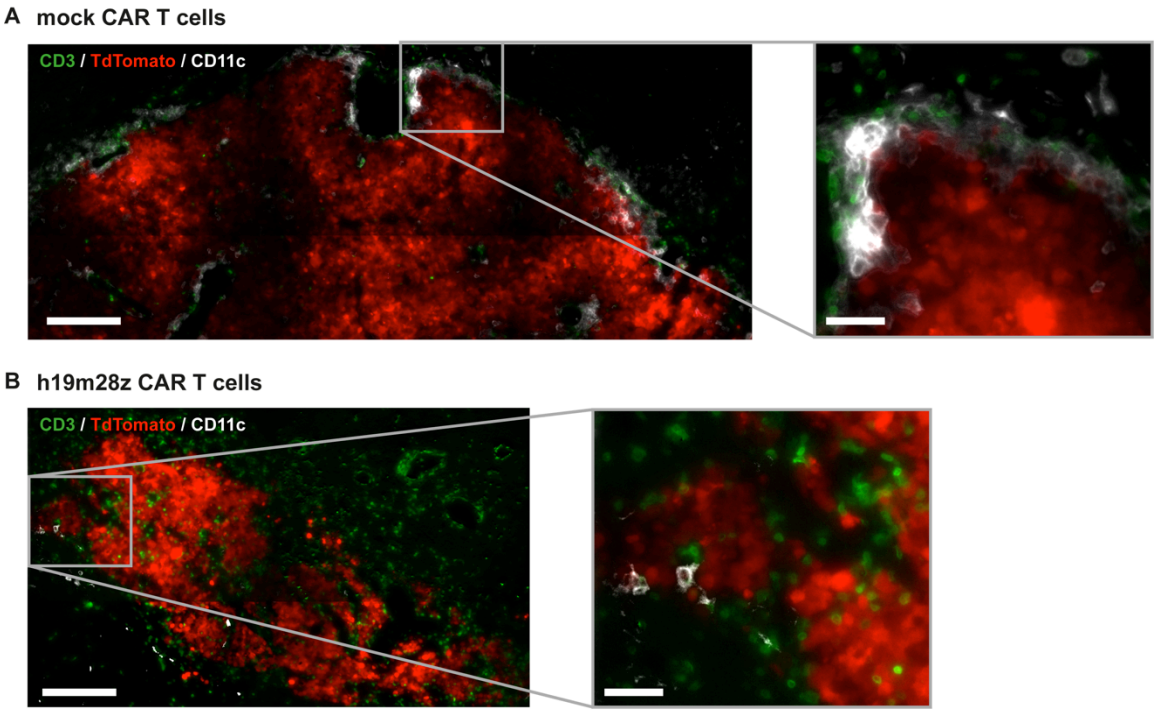


**Fig. S10. CD11c$^+$ cells surround PCNSL and are in close contact to CD3$^+$ T cells after mock CAR T cell injection, whereas CD11c$^+$ cells are found scarcely around intracerebral lymphoma in h19m28z CAR T cell-treated mice.** Immunofluorescence showing U2932 cells (tdTomato, red) and CD11c$^+$ myeloid cells (gray) 28 days after extratumoral, intracerebral mock CAR T cell injection (**A**) or h19m28z CAR T cell injection (**B**). Scale bars, 200 µm (left) and 25 µm (inlays, right). Representative samples of two mice per group of one independent experiment is presented.

**Movie S1.** Intratumoral h19m28z CAR T cell distribution 14 days after intracerebral CAR T cell injection.

**Movie S2.** Intratumoral mock CAR T cell distribution 14 days after intracerebral CAR T cell injection.

**Movie S3.** Intratumoral h19m28z CAR T cell movement and their intratumoral migration tracks. Time-lapse video of 30 minutes duration.

**Movie S4.** Intratumoral h19m28z CAR T cell mitosis. Time-lapse video of 23 minutes duration.

**Movie S5.** Intracranial h19m28z CAR T cells 98 days after intracerebral injection without tumor recurrence. Time-lapse video of 30 minutes duration.

**Movie S6.** Intracranial h19m28z CAR T cell 110 days after intracerebral injection without tumor recurrence. Time-lapse video of 5 minutes duration.

**Movie S7**. Intracranial and intravascular h19m28z CAR T cells 159 days after intracerebral injection demonstrating rolling of CAR T cells along the vessel wall. Time-lapse video (CCD camera) of 5 seconds duration.

**Movie S8.** Intracranial and intravascular h19m28z CAR T cells. Time-lapse video (CCD camera) of 5 seconds duration.