Peripherally induced brain tissue-resident memory CD8\(^+\) T cells mediate protection against CNS infection

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The central nervous system (CNS) is classically viewed as immune-privileged; however, recent advances highlight interactions between the peripheral immune system and CNS in controlling infections and tissue homeostasis. Tissue-resident memory (TRM) CD8\(^+\) T cells in the CNS are generated after brain infections, but it is unknown whether CNS infection is required to generate brain TRM cells. We show that peripheral infections generate antigen-specific CD8\(^+\) memory T cells in the brain that adopt a unique TRM signature. Upon depletion of circulating and perivascular memory T cells, this brain signature was enriched and the surveilling properties of brain TRM cells was revealed by intravital imaging. Notably, peripherally induced brain TRM cells showed evidence of rapid activation and enhanced cytokine production and mediated protection after brain infections. These data reveal that peripheral immunizations can generate brain TRM cells and will guide potential use of T cells as therapeutic strategies against CNS infections and neurological diseases.

The CNS has been viewed as an immunologically privileged site and the presence of T cells in the CNS considered a pathogenic condition. These concepts stem from ideas that the CNS lacks classical lymphatics, the blood–brain barrier limits immune cell trafficking and CNS-residing cells express little major histocompatibility (MHC) protein. However, recent discoveries of meningeal lymphatics and increasing roles of T cells in normal homeostatic functions of the CNS are changing our perception of this immunologically privileged concept. A role for T cells in the CNS is now described in brain development, protection against CNS injury, neurodegeneration and control of CNS infections.

Infections with intracellular pathogens induce memory CD8\(^+\) T cells, enabling effective secondary immune responses to the same pathogen. Memory CD8\(^+\) T populations consist of central memory (T\(_{CM}\)), effector memory (T\(_{EM}\)) and T\(_{RM}\) cells. T\(_{RM}\) cells are distinct from T\(_{EM}\) and T\(_{RM}\) cells in that they do not recirculate but instead reside in tissues. T\(_{RM}\) cells populate sites of initial infection or vaccination and are characterized in barrier tissues (for example skin, intestine, female reproductive tract (FRT) and lungs), lymphoid (for example spleen and lymph nodes) and peripheral tissues (for example kidneys, liver and brain). Although heterogeneity in T\(_{RM}\) populations exists, several markers of tissue residence, including cellular adhesion molecules (integrins) CD49a, CD11a and CD103, chemokine receptors CXCR3 and CXCR6 and the tissue retention marker CD69 (ref. 11) distinguish them from circulating memory subsets. Multiple studies suggest T\(_{RM}\) cells protect from secondary infections by direct killing of infected target T cells, recruitment of additional immune cells to sites of infection and by creation of tissue antiviral states.

Memory CD8\(^+\) T cells can contribute to control of CNS infections. However, it remains unknown whether and how each subset contributes to CNS immunity. Early studies of intranasal (i.n.) infection with the neurotropic vesicular stomatitis virus (VSV) identified CD103\(^+\) T\(_{RM}\) cells in the CNS. Furthermore, characterization of VSV and Toxoplasma gondii–induced T\(_{RM}\) cells in the brain identified a key transcriptional signature of CD103\(^+\) brain CD8\(^+\) T cells compared to CD103\(^-\) splenic CD8\(^+\) T cells. Additionally, CNS T\(_{RM}\) cells are induced after intracranial (i.c.) injections with recombinant lymphocytic choriomeningitis virus (rLCMV), polyomavirus (PyV) and murine cytomegalovirus (MCMV). While most antigen-specific cells isolated from the brain after CNS infections expressed CD69, only some (~20–80%, depending on the model) were also CD103\(^+\) (refs. 14,15,17,18,20). A common theme of these studies, which all rely on CNS infection, is the suggestion that antigen expression in the CNS is required for generation of T\(_{RM}\) CD8\(^+\) T cells in this tissue.

T\(_{RM}\) cells are described in most human tissues, including the brains of autopsy patients. The antigen specificity of T\(_{RM}\) cells in human brains is unknown, but they are speculated to be specific for neurotropic pathogens. Naive mice housed in specific-pathogen-free conditions harbor detectable CD8\(^+\) T cells in the brain, which increase in numbers in aged mice and more recently, single-cell analysis revealed oligoclonal expansion of T cells in the brains of old mice in the absence of overt infection. Another study identified oligoclonal CD8\(^+\) T cells patrolling the cerebrospinal fluid in human patients. Together, these data suggest that T cell surveillance in the CNS may occur in the absence of direct brain infection. Here, we used mouse models to probe the characteristics of CNS CD8\(^+\) T cells in young mice after brain infection and compare them to CNS CD8\(^+\) T cells generated after peripheral infections with diverse pathogens or after immunization in the absence of infection.

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**Results**

**Brain CD8+ T cells induced after peripheral infection.** Brain CD8+ T cells are generated after CNS infection in multiple mouse models3 but it is unknown whether CNS infections are required to generate these cells. To test this, we compared CNS TRm cell generation in mice infected i.n. with the vesicular stomatitis virus expressing ovalbumin (VSV-OVA), which causes brain infection or a range of peripheral infections. Mice were seeded with enhanced green fluorescent protein (eGFP)-expressing OT-I or P14 T cells from naive donors and infected with the indicated pathogens expressing OVA257–264 or GP33–41. Before brain collection at a memory time point (>25d post-inoculation) mice were injected intravenously (i.v.) with anti-CD45 fluorophore-conjugated antibody (IV stain) for 3 min to discriminate cells within the vasculature (IV+) or within the tissue (IV-) and splenocytes (SP), peripheral blood (PBL) and brain lymphocytes were analyzed. IV+ and IV- OT-I cells were present in the brain of mice infected i.n. with VSV-OVA, with an increased representation of OT-I cells appearing in IV- brain tissue compared to the brain vasculature (IV+), the SP and PBL (Fig. 1a,b and Extended Data Fig. 1a). Thus, antigen-specific memory CD8+ T cells were enriched in brain tissues after CNS infection4.

In contrast to VSV, influenza A (IAV) replication is generally restricted to lung epithelial cells after i.n. infection but this does not mean that virus or antigen has no access to the CNS. To determine whether i.n. IAV infection induced enrichment of memory CD8+ T cells in the CNS, mice were seeded with P14 CD8+ T cells and inoculated with a non-neurotropic IAV/PR8/34 expressing GP33 (IAV-GP33). Memory P14 CD8+ T cells were found at an increased proportion in the IV- brain lymphocyte fraction (Fig. 1c). Thus, it was possible that the i.n. route of infection enhanced T cell entry into brain tissues.

Replication of VSV in the CNS occurs after i.n. or i.c. inoculation, but does not occur during i.v. or intraperitoneal (i.p.) inoculations. Similarly, i.v. or i.p. infections with lymphocytic choriomeningitis virus Armstrong strain (LCMV Arm) do not result in extensive CNS infections5. Despite this, i.p. VSV-OVA and i.p. or i.v. LCMV Armstrong strain (LCMV Arm) do not result in extensive CNS infection28. Similarly, i.v. or i.p. infections with lymphocytic choriomeningitis virus Armstrong strain (LCMV Arm) do not result in extensive CNS infection. However, it is impossible to rule out low CNS infections in all models tested. To address this, we immunized OT-I recipient mice by DC-OVA priming followed by bacterial infection with recombinant Listeria monocytogenes expressing ovalbumin (rLM-OVA) and after OVA peptide-coated dendritic cell (DC) prime-rLM-OVA boost-immunization (DC-OVA-rLM-OVA, both delivered i.v.) (Fig. 1f,g). Similar results were obtained when targeting endogenous OVA-specific populations with DC-OVA-rLM-OVA immunization, P14 cells with DC-GP33-rLM-GP33 immunization or after DC-OVA prime-vaccinia virus (VV)-OVA boost (Extended Data Fig. 1c-e). Finally, we extended our analyses to rLM-OVA IV- infected outbred NIH Swiss Webster mice. The proportion of CNS CD8+ T cells, IV- CD8+ T cells and total number if IV- CD8+ T cells were all increased in mice infected with rLM-OVA compared to naive controls (Extended Data Fig. 1f).

Our results showed that pathogen-specific memory CD8+ T cells were enriched in brains after peripheral infections that are not generally thought to cause CNS infections. However, it is impossible to rule out low CNS infections in all models tested. To address this, we immunized OT-I recipient mice by DC-OVA priming followed by boosting with naive splenocytes coated with the OVA peptide, a scenario leading to robust CD8+ T cell peripheral responses without infection. Again, we found memory OT-I cells to be enriched in the IV- brain fraction (Fig. 1h). These data definitively showed that enrichment of memory CD8+ T cells in brain tissue did not require CNS infection.

To determine how long peripherally induced memory cells in the CNS persisted, proportions of OT-I cells after DC-OVA-rLM-OVA prime-boosting were evaluated over time in the SP, PBL and IV- brain (Fig. 1i). Increased proportions of OT-I cells in IV- brain tissues persisted up to 1 year post-inoculation, indicating population longevity in the CNS. In summary, these data demonstrated that peripheral infections with diverse pathogens or immunizations resulted in long-term maintenance of antigen-specific memory CD8+ T cells in the brain.

**Brain CD8+ T cells adopt a TRm phenotype.** Memory T cells may transiently enter tissues or persist long-term as TRm cells. To address the gene expression profile of peripherally induced brain CD8+ T cells, RNA-sequencing (RNA-seq) was performed on memory OT-I cells isolated from the SP, IV+ and IV- brain of DC-OVA-rLM-OVA immunized mice. Approximately 1,800 genes were differentially expressed between brain IV+ and IV- OT-I cells (Fig. 2a). Of note, TRm cells from other tissues have characteristic core gene expression profiles35,36,37. We compiled a list of 239 such genes and assessed their relative expression in SP, IV+ and IV- brain OT-I cells. Genes encoding several transcription factors (Klf2, Klf3, Zfp683 and Bhlhe40)35,36,37 and phenotypic markers (CX3CR1, Lkr1, S1PR1, CXCR3 and CX69)31,32,33, all associated with tissue residence, were differentially expressed in the IV- brain compared to either SP or IV+ brain (Fig. 2b and Extended Data Fig. 2a,b). These data suggested that peripherally induced IV- brain CD8+ T cells may be transcriptionally similar to tissue infection-induced TRm cells.

Several makers including CD69 and CD103 have been used to characterize the phenotype of brain TRm after CNS infections34. To address the phenotype of peripherally induced brain TRm cells, we used spanning-tree progression analysis of density normalized events (SPADE) analysis, a nonbiased approach to analyze flow cytometric data and to separate cells with similar characteristics into nodes of different sizes representing different number of cells. Surface expression of CD8a, CD11a, CD44, CD49a, CD69, CD103, CXCR6 and CXCR1, were used for initial SPADE analysis to identify characteristics of memory OT-I cells from the DC-OVA-rLM-OVA immunized mouse brains. SPADE analysis was initially performed to generate a dendrogram of all OT-I cells in the brain without considering the IV+ or IV- status (Fig. 2c). Next, IV- or IV- OT-I cells were gated and SPADE analysis was performed with the indicated markers on each population to generate dendograms (Fig. 2c). SPADE analysis identified two distinct clusters of cells that clearly segregate the IV+ and IV- populations isolated from the CNS without using IV exclusion as an a priori marker (Fig. 2c).

Additional phenotyping of peripherally induced brain CD8+ T cells identified a pattern resembling TRm cells rather than TCM or TRm populations (Fig. 2d). Peripherally induced brain CD8+ T cells had high CD69 expression and a small subset expressed CD103. Brain IV+ OT-I cells also showed increased CD8a expression, similarly to brain TRm CD8+ T cells described in other tissues35,37. Chemokine receptors CXCR3 and CXCR6 that are characteristic of TRm cell subsets in other organs were also increased in IV- brain CD8+ T cells. TRm cells in other tissues express low amounts of CD62L, CD122 and KLRC1 and peripherally induced brain IV- CD8+ T cells also showed reduced expression of these molecules11. The glycosylated form of CD43 has been associated with T cell activation and trafficking and was increased in CD8+ T cells from the IV- brain compared to IV+ brain compartments. Brain IV- CD8+ T cells showed reduced expression of CX3CR1 compared to IV+ CD8+ T cells, suggestive of a tissue residing9,10 but not a circulating population. Combined, these data indicated that upon peripheral infection, antigen-specific CD8+ T cells that enter the CNS display a substantially different phenotype from those in the brain vasculature and this phenotype is similar to canonical TRm cells rather than TCM or TRm cells.
Fig. 1 | Enrichment of antigen-specific CD8\(^+\) T cells in the CNS after peripheral immunizations. a. Representative flow cytometry plots of brain samples isolated from mice seeded with eGFP\(^+\) OT-1 CD8\(^+\) T cells and infected with VSV-OVA i.n. Representative of three independent experiments with four mice per group. b–i. The proportion of donor OT-1 or P14 cells of live CD8\(^+\) T cells isolated from SP, PBL, IV+ brain (IV+) or IV– brain (IV–) are graphed from memory >30 d or the indicated days after infection with VSV-OVA i.n., \(n=4\), from three independent experiments; \(P\) values (top to bottom) are \(*\)* =\( P<0.0001\), \(**\) =\( P<0.001\), \(**\) =\( P<0.0001\), \(***\) =\( P=0.003\), \(****\) =\( P=0.0004\); OT-1 of CD8\(^+\) (b); IAV-GP33 i.n., \(n=4\), from two independent experiments; \(P\) values (top to bottom) \(*\)* =\( P<0.0001\), \(**\) =\( P<0.001\), \(**\) =\( P<0.0001\), \(***\) =\( P=0.0002\); VSV-OVA i.p., \(n=4\), from three independent experiments; \(P\) values (top to bottom) \(*\)* =\( P<0.0001\), \(**\) =\( P<0.001\), \(**\) =\( P<0.0001\), \(***\) =\( P=0.003\), \(****\) =\( P<0.0001\); LCMV i.p., \(n=3\), from three independent experiments; \(P\) values (top to bottom) \(*\)* =\( P<0.0001\), \(**\) =\( P<0.001\), \(**\) =\( P<0.0001\), \(***\) =\( P=0.006\), \(P=0.0169\); LCMVArm i.p., \(n=3\), from three independent experiments; \(P\) values (top to bottom) \(*\)* =\( P<0.0001\), \(**\) =\( P<0.001\), \(**\) =\( P<0.0001\), \(***\) =\( P<0.0001\), \(****\) =\( P<0.0001\); DC-OVA-rLM-OVA i.v., \(n=4\), from two independent experiments; \(P\) values (top to bottom) \(*\)* =\( P<0.0001\), \(**\) =\( P<0.001\), \(**\) =\( P<0.0001\), \(***\) =\( P=0.0006\), \(P=0.0006\); DC-OVA–rLM-OVA prime-boost i.v., \(n=3\), from four independent experiments; \(P\) values (top to bottom) \(*\)* =\( P<0.0001\), \(**\) =\( P<0.001\), \(**\) =\( P<0.0001\), \(***\) =\( P<0.0001\), \(****\) =\( P<0.0001\), \(****\) =\( P<0.0001\); VSV-OVA i.p., \(n=4\), from three independent experiments; \(P\) values (top to bottom) \(*\)* =\( P=0.0087\), \(**\) =\( P=0.0083\), \(***\) =\( P=0.0183\); or DC-OVA–rLM-OVA i.v., \(n=3\), 4, 2, 3 and 3 at each respective time point (left to right) (f), p.i., post-immunization. Graphs show the mean ± s.d. (b–h) or mean ± s.e.m. (i) with each dot representing an individual mouse. Statistical significance was determined by one-way analysis of variance (ANOVA) with Tukey’s multiple comparisons post-test using GraphPad Prism with individual \(P\) values reported between groups.

Previous studies identifying brain T\(_{BM}\) populations induced by CNS infections have described a wide range of CD103 expression\(^{20,14,13,17,18,22}\). Peripherally induced brain T\(_{BM}\) cells showed some CD103 expression and therefore we questioned whether CD103 was required for their generation, as has been shown in other T\(_{BM}\) populations\(^{18}\). CD103KO mice generated similar numbers of IV+ CD69\(^+\) OVA-tetramer-positive cells in the CNS compared to wild-type mice after DC-OVA-rLM-OVA immunization (Extended Data Fig. 2c). Moreover, adoptively transferred CD103KO P14 cells generated increased proportions of donor cells in the IV+ brain after LCMV i.p. infection, in a pattern similar to wild-type P14 cells (Extended Data Fig. 2d,e). Together, these data suggested that CD103 expression is not absolutely required for generation of peripherally induced brain T\(_{BM}\) cells.

In addition to phenotypic differences in T\(_{BM}\) compared to T\(_{CM}\) or T\(_{EM}\) cells, several transcription factors such as KLF2, BLIMP-1, Hobit and Runx3 (refs. 29–32) have been associated with the development of T\(_{BM}\) populations. Indeed, KLF2 mRNA was substantially reduced in IV+ brain T cells compared to IV+ brain T cells, whereas Pdml1 (encoding BLIMP-1) and Zfp683 (encoding Hobit) were modestly upregulated. In contrast, Runx3 was not differentially expressed between IV+ and IV– brain T cell populations. However, Runx3 clearly has a positive role in generation of T\(_{BM}\) cells in other tissues\(^{20}\) and Runx3-deficient P14 cells, although exhibiting an overall reduction in the magnitude of response, were not enriched in the IV+ brain when compared to the IV– compartment (Extended Data Fig. 2f,g). These data suggested a critical role for Runx3 in the generation of peripherally induced brain T\(_{BM}\) cells.
Brain T_RM cells display unique signatures. T_RM, CD8^+ T cells are generally described at the site of initial infection; therefore, it was unexpected to find T_RM cells enriched in the CNS after peripheral infections or immunizations. To determine whether peripheral infection-induced enrichment of T_RM cells was unique to the CNS, naive OT-I cell-bearing mice were immunized i.v. with rLM-OVA and memory OT-I cells were analyzed from tissues including PBL, SP, liver, kidney, lung and brain. Increased proportions of OT-I cells were only found in the IV^‘ brain (Fig. 3a). Furthermore, SPADE analysis, using the same makers described in Fig. 2c on IV^‘ OT-I cells from all of these tissues, identified a phenotypic cluster of cells that were enriched the brain compared to other organs tested.

To determine whether this brain T_RM cell phenotypic cluster was unique to peripheral *Listeria* infection, memory P14 cells stimulated by i.p. LCMV infection were analyzed in multiple organs. Again, P14 CD8^+ T cells were enriched in the IV^‘ brain and not in the other organs (Fig. 3c). SPADE analysis, using the same makers described previously, also identified a brain specific phenotype of IV^‘ P14 cells that was not found in IV^‘ T cell populations in other organs despite known LCMV replication in peripheral organs, including SP, liver and kidney (Fig. 3d). Similarly to *Listeria* infection, SPADE analysis of LCMVArm infection identified 89% of cells in a phenotypic cluster from the brain but this population was not prevalent in other organs tested.

Memory CD8^+ T cell populations with distinct phenotypes are generated in response to different infectious agents, routes of pathogen administration and time after memory generation. To address these issues, IV^‘ OT-I cells in 1-month-rLM-OVA-infected mice (early) were compared to IV^‘ P14 cells in 5-month-LCMVArm-infected mice (late). As expected, IV^‘ antigen-specific cells from the SP showed very distinct population dendrograms, with nodes from each infection separating into different clusters (Fig. 3e). However, the brain IV^‘ antigen-specific donor cells clustered together in similar nodes in response to either pathogen, suggesting that there was a signature of CD8^+ T cells in the brain that was induced irrespective of the peripheral infection used. Combined, these data suggested that peripheral infections induced a population of T_RM cells in the CNS that were phenotypically distinct from T_RM cells from other peripheral organs.

To this point, our analyses focused on whole brains, which include brain parenchyma and meninges. To distinguish these compartments, we used a physical separation approach. DC-OVA-rLM-OVA immunizations increased OT-I frequencies in the IV^‘ fraction from both brain and meninges compared to the PBL (Extended Data Fig. 3a,b). Moreover, the phenotype of OT-I memory cells isolated from meninges very closely resembled that expressed by OT-I cells isolated from the brain parenchyma (Extended Data Fig. 3c–g), illustrated by the clustering of >99% of the IV^‘ OT-I populations from the brain and the meninges after SPADE analysis (Extended Data Fig. 3h). Combined, these data suggested that after peripheral immunization, the antigen-specific CD8^+ T_RM population in the CNS was distinct from that of other peripheral organs.

**Dynamics of CD8^+ T cells in the CNS.** Intravital microscopy can provide valuable information about the behavior and potential function(s) of cell populations in tissues. For example, CD8^+ T_RM cells in the skin are mostly arrested (<2 μm min^‘1^) compared to those in the FRT that showed much faster average speeds of around 10 μm min^‘1^ (ref. 44). To address cellular dynamics of peripherally induced brain T_RM cells, we immunized mice with DC-OVA-rLM-OVA and performed intravital imaging of brain memory eGFP^‘ OT-I cells to visualize cells in the meninges and superficial cortical layers (Supplemental Movies 1, 2 and Fig. 4a). Brain OT-I cells exhibited varying dynamic properties, including moving large distances over short periods of time, crawling along the outside of the vessels, remaining mostly stationary with some tugging back and forth and even interacting with other OT-I cells. The majority (62%) of peripherally induced memory OT-I cells in the brain were traveling between 2 and 5 μm min^‘1^, whereas 15% remained arrested (<2 μm min^‘1^) and 23% of cells were traveling with speeds greater than 5 μm min^‘1^.

Peripherally induced T cell populations persisted long-term in the CNS, therefore, we determined whether the dynamics of memory CD8^+ T cells change from early memory (1–2 months post-inoculation) to late memory (~16 months post-inoculation). No discernable differences were observed between these groups for average speed (Fig. 4b) and arrest coefficients (Fig. 4c). Given the observation that a wide range of cellular dynamic properties were seen both at early and late memory time points, we questioned whether a particular type of behavior was associated with anatomical location (perivascular versus parenchymal). To address this, memory Thy1.1^‘eGFP^‘ OT-I cells were generated by DC-OVA-rLM-OVA immunization and intravital imaging of the brains was performed. At 30 min before imaging, mice were injected i.v. with anti-Thy1.1-PE to identify cells capable of being labeled i.v., specifically those in perivascular spaces (Supplemental Movies 3 and 4). Consistent with perivascular localization, double-positive (Thy1.1^‘eGFP^‘) OT-I exhibited reduced average speeds and increased arrest coefficients compared to Thy1.1^‘eGFP^‘ single-positive cells (Fig. 4c). These data suggested that memory CD8^+ T cells in the brain parenchyma display a vigorous patrolling movement pattern.

**Brain CD8^+ T cells resist systemic depletion.** Bona fide T_RM populations in the skin, FRT and other organs are resistant to systemic antibody-mediated depletion, a characteristic of their tissue residence. To address this, memory Thy1.1^‘ OT-I cells in the brain were induced by DC-OVA-rLM-OVA immunization and mice were injected with various doses of Thy1.1-depleting antibody. One week
post treatment, OT-I cells were depleted in the SP, PBL and in the IV+ brain; however, the IV- OT-I cells in the brain were protected from antibody depletion (Extended Data Fig. 4a and Fig. 5a,b).

To address whether peripherally induced brain T_{RM} cells require a circulating population for maintenance, organs were collected 1 month after antibody depletion. IV- OT-I cells were similarly
maintained both in the presence and absence of peripheral OT-I responses, (Fig. 5c) indicating peripherally induced brain T<sub>RM</sub> cells persist in the absence of a circulating memory pool. To assess whether the phenotype of the CD8<sup>+</sup> T cell population in the brain changed upon systemic depletion with antibody, SPADE analysis, using the aforementioned markers, was performed using...
IV-OT-I cells from control-treated mice as the comparator to each of the antibody depletion doses tested (Fig. 5d). Two major clusters (upper and lower) were identified in control mice. The percentage of IV- antigen-specific cells in the upper cluster was enriched after peripheral depletion in an antibody dose-dependent manner. Thus, although the proportion and number of OT-I cells in the IV-CNS did not seem to differ between groups (Fig. 5a,b), there was enrichment in a specific phenotypic cluster upon systemic antibody depletion. Indeed, analysis of individual markers determined that CD69 (Extended Data Fig. 4b), CD103, CD49a and CXCR6 (data not shown) expression were elevated in the IV-OT-I cells remaining after systemic antibody depletion. This suggested that two distinct populations in the IV-CNS exist: a resident population that was resistant to systemic depletion and a population that was susceptible to systemic depletion.

Next, we sought to determine whether T cell dynamics were also altered by systemic depletion. Indeed, the average (Fig. 5c) and maximum speed (Extended Data Fig. 4c) were increased, whereas the arrest coefficients decreased (Fig. 5f) in brain OT-I cells from systemically depleted mice compared to control mice. These data suggested that the variable cellular dynamic properties shown in Fig. 4 and in nondepleted mice in Fig. 5 resulted from brain antigen-specific CD8+ T cells consisting of both perivascular or transient IV- cells (more susceptible to being depleted) and resident parenchymal populations, whose movement dynamics may facilitate CNS surveillance.

Brain T_{res} cells protect against CNS infections. In previous studies, T_{res} cells in multiple organs exhibited increased effector cytokine production compared to vascular or non-T_{res} tissue-derived...
CD8+ T cells\(^1,2,3\). Increased expression of Il2, Ifng and Tnf genes was identified in our brain T\(_{\text{RM}}\) cells compared to T cells from SP in our unbiased RNA-seq analysis. Consistent with this, brain IV+ memory OT-I T cells showed enhanced single and multiple cytokine-producing capabilities compared to IV+ OT-I T cells (Fig. 6a–c). Similarly, DC-GP33-rLM-GP33-induced brain memory IV+ P14 cells showed increased capacity to produce multiple cytokines compared to IV+ P14 cells (data not shown). These data show that peripherally induced brain CD8+ T\(_{\text{RM}}\) cells also exhibit enhanced cytokine-producing capabilities.

Increased effector function during in vitro stimulation does not fully indicate enhanced responses against pathogens in vivo. Two different experimental approaches were utilized to address whether peripherally induced brain T\(_{\text{RM}}\) cells were capable of rapid responses to infection. Memory OT-I brain T\(_{\text{RM}}\) cells and circulating memory cells were generated via DC-OVA-VACV-OVA immunization and mice either were unchallenged or challenged with rLM-OVA i.c. OT-I cells in the brain, but not other tissues, upregulated CD25 (Extended Data Fig. 5a) and produced interferon (IFN)-γ (Extended Data Fig. 5b) when isolated 2 d after challenge. Similarly, brain IV+ P14 cells induced by LCMV infection upregulated CD25 (Fig. 6f,g) and produced IFN-γ (Fig. 6h,i) after i.c. challenge with rLM-GP33 and this was not impacted by peripheral depletion (Extended Data Fig. 5c,d) nor observed in circulating memory CD8+ T cells in non-depleted mice. Additionally, T\(_{\text{RM}}\) cells have the potential to recruit circulating cells to the site of challenge\(^4\). Consistent with this property, total P14 cells were increased in LCMV immunized mice after i.c. challenge with rLM-GP33 compared to the numbers recovered after i.c. challenge of immunized mice that had been depleted of circulating P14 cells (Extended Data Fig. 5e).

T\(_{\text{RM}}\) cells control secondary infections by killing infected cells, inducing an antiviral state in the reinoculated tissue and producing

**Fig. 5** | Peripherally induced CNS T\(_{\text{RM}}\) cells are resistant to systemic depletion. a,b, Thy1.1 eGFP+ OT-I T cells were transferred into naive mice that were DC-OVA-rLM-OVA prime-boosted. After memory formation, mice were treated with control (PBS) or 2, 5 or 10 \(\mu\)g of anti-Thy1.1 i.p. and, 1 week after depletion, phenotype and numbers of donor cells were determined. Proportion of OT-I cells in SP, PBL, IV+ brain and IV+ brain of CD8+ T cells are graphed (a). Number of IV- OT-I cells in brain are graphed (b). Data are representative of two independent experiments with three mice per group. Graphs show the mean±s.d. with each dot representing an individual mouse. c, Thy1.1 eGFP+ OT-I T cells were transferred into naive mice that were DC-OVA-rLM-OVA prime-boosted. After memory formation, mice were treated with control (PBS) or 2 \(\mu\)g of anti-Thy1.1 i.p. At 1 month after depletion, phenotype and numbers of donor cells were determined. Proportion of OT-I cells of CD8+ T cells are graphed (c). Data are representative of two independent experiments with three mice per group. Graphs show the mean±s.d. with each dot representing an individual mouse. d, Thy1.1 eGFP+ OT-I T cells were transferred into naive mice that were DC-OVA-rLM-OVA prime-boosted. After memory formation, mice were treated with control (PBS) or 2, 5 or 10 \(\mu\)g of anti-Thy1.1 i.p. and, 1 week after depletion, phenotype and numbers of donor cells were determined. Proportion of OT-I cells in SP, PBL, IV+ and IV- cells are graphed (d). Data are representative of two independent experiments with three mice per group. Graphs show the mean±s.d. with each dot representing an individual mouse. e, Thy1.1 eGFP+ OT-I T cells were transferred into naive mice that were DC-OVA-rLM-OVA prime-boosted. After memory formation, mice were treated with control (PBS) or 2, 5 or 10 \(\mu\)g of anti-Thy1.1 i.p. At 1 week after depletion, OT-I cells in the CNS were imaged and average cell speed (e) and arrest coefficients (f) were determined. Graphs show the mean±s.d. with each dot representing an individual cell, analyzing 71 cells from 12 individual movies (PBS) and 103 cells from 16 individual movies (2 \(\mu\)g). Statistical significance was determined by two-sided Mann-Whitney U-test using GraphPad Prism, where ****\(P<0.0001\) (e) and ****\(P<0.0001\) (f).
cytokines that facilitate the recruitment of other immune cells to the site through changes to the vascular endothelium and local chemokine milieu. TRM cells generated by direct CNS infection are thought to provide enhanced resistance to brain reinfection but it is unknown whether peripherally induced brain TRM cells contribute to immunity or pathogenesis after reinfection. To address this, control and DC-OVA-rLM-OVA immunized mice with peripherally induced brain CD8+ TRM cells were challenged with VSV-OVA
Fig. 7 | Peripherally induced brain-resident CD8+ T cells mediate protection against CNS infections. **a.** Thy11 OT-I cells were transferred into mice and recipients were DC-OVA-rLM-OVA prime-boosted. After memory formation, these mice and naive controls were treated with control (PBS) or 2 μg anti-Thy1.1 i.p. and challenged with VSV-OVA i.c. 1 week after depletion. VSV-OVA titers at 3 d after challenge are graphed. Data are combined from two independent experiments for a total of 10, 8 and 9 mice per group (left to right). Graph shows the mean ± s.d. with each dot representing an individual mouse. Statistical significance was determined by one-way ANOVA with Tukey’s multiple comparisons test across all the groups using GraphPad Prism with P values (top to bottom) of **P = 0.0035 and ***P = 0.0022. **b.** Thy11 P14 cells were transferred into mice and recipients were infected with LCMVArm i.p. After memory formation, mice were treated with control (PBS) or 2 μg anti-Thy1.1 i.p. and these mice and naive controls were challenged with rLM-GP33 i.c. 1 week after depletion. Bacterial colony-forming units (c.f.u.) per gram of brain are plotted at 2d after challenge. Data are combined from two independent experiments for a total of ten mice per group. Graph shows the mean with each dot representing an individual mouse. Statistical significance was determined by one-way ANOVA with tukey’s multiple comparisons test across all the groups using GraphPad Prism with P values (top to bottom) of **P < 0.0001 and ****P < 0.0001. **c.** Thy1 OT-I cells were transferred into mice and recipients were DC-OVA-VACV-OVA prime-boosted. After memory formation, mice were treated with control (PBS) or FTY720 on days −4, 2 and 0 before challenge with rLM-OVA i.c. Bacterial c.f.u. per gram is plotted at 2d after challenge. Data are combined from two independent experiments for a total of 7, 12 and 8 (left to right) mice per group. Graph shows the mean with each dot representing an individual mouse. Statistical significance was determined by one-way ANOVA with Tukey’s multiple comparisons test across all the groups using GraphPad Prism with P values (top to bottom) of ****P < 0.0001 and ****P < 0.0001. **d.** Thy11 OT-I or P14 cells were transferred into mice and DC-OVA-rLM-OVA or DC-GP33-rLM-GP33 prime-boosted, respectively. After memory formation, mice were treated with control (PBS) or 2 μg anti-Thy1.1 i.p. and challenged with LCMV i.c. 1 week after depletion. Kaplan–Meier survival curves (**d**) and weight loss curves (**e**) are depicted for LCMV i.c. only controls (black, n = 10), DC-OVA-rLM-OVA no depletion (red, n = 13), DC-OVA-rLM-OVA with anti-Thy1.1 (yellow, n = 5), DC-GP33-rLM-GP33 no depletion (navy, n = 10) or DC-GP33-rLM-GP33 with anti-Thy1.1 (blue, n = 10). Data are combined from two independent experiments for a total of 5–13 mice per group. GraphPad Prism was used to determine significance using a Mantel–Cox test for each group comparing to the LCMV i.c. only challenge group (**d**), where P values are ****P < 0.0001 (navy) and ****P < 0.0001 (blue).

i.n. Three days after infection, immune mice exhibited reduced viral titers compared to controls, albeit the range in titers was broad (Extended Data Fig. 6a), likely due to the variability with the i.n. route of infection. To examine a more reproducible model, control and DC-OVA-rLM-OVA immune mice were generated, some mice were depleted of circulating OT-I and all mice were challenged with VSV-OVA i.c. Control mice had higher viral titers (Fig. 7a) and died earlier (Extended Data Fig. 6b) than immune mice containing brain T BM OT-I cells. Of note, protection did not require circulating memory OT-I cells, suggesting that the brain-resident T BM cells were capable of independent antiviral activities. Similarly, mice containing brain Thy1.1 P14 cells generated by i.p. LCMVA rm infection exhibited reduced bacterial burden compared to control mice after rLM-GP33 i.c. challenge (Fig. 7b) and protection did not require circulating memory P14. Additionally, DC-OVA-VACV-OVA immune mice treated with FTY720 to inhibit cellular ingress (and egress) from other organs controlled rLM-OVA infection similarly to vehicle-treated immune controls (Fig. 7c). Combined, these data suggested that peripherally induced brain-resident memory cells can contribute to pathogen control with and without recruiting antigen-specific cells from the periphery.

Potent CD8+ T responses in some models of viral encephalitis are pathogenic rather than protective, as in the case of meningitis induced in adult mice by i.c. infection with LCMVA rm. To address a role for brain T BM OT-I cells in LCMV-induced meningitis, mice were seeded with Thy1.1 T cell receptor (TCR)-transgenic (tg) T cells and were primed against OVA (containing OT-I cells) or GP33 (containing P14 cells) and some mice were depleted of peripheral memory cells (Extended Data Fig. 6c,d). Regardless of depletion, mice containing nonspecific brain T BM OT-I cells succumbed to i.c. LCMV infection with similar kinetics as naive controls (Fig. 7d,e). However, mice containing GP33-specific brain T BM OT-I cells, regardless of whether peripheral P14 cells were depleted, were protected from LCMV-induced morbidity and mortality compared to controls. Combined, these data suggested that peripherally induced T BM cells in the brain can provide protection in several models of brain infection by limiting pathogen replication and reducing morbidity and mortality.
Discussion
Historically, it was thought that the only time T cells entered the CNS was to control infections (often with adverse consequences) or contribute to CNS autoimmune diseases. However, our present understanding of T cells in the brain indicates that they contribute to brain development, protect against CNS injury and neurodegeneration and aid in controlling infections1. As shown here, peripheral immunizations uniformly increase antigen-specific CD8+ T RM cells in the CNS and these cells can limit brain infections. These results support emerging concepts that the CNS may be a more welcoming environment for T cells than previously appreciated.

Antigen-specific CD8+ T cells seed the brain after peripheral immunizations; however, the route these cells utilize to enter the CNS remains unknown. The CNS has multiple barrier structures to limit inflammation and cellular infiltration into the parenchyma, but these barriers can also serve as entry points for pathogens and leukocytes and may be altered by systemic inflammation1. Alternatively, although most of the CNS is protected from peripheral infiltration by the blood–brain barrier, some locations, such as the choroid plexus, have less restrictive barriers where T cells have been shown to accumulate in response to superantigen stimulation24. A recent study determined that neurogenic niches contained oligoclonal T cells with T RM gene expression profiles and also found T cells throughout the brain in aged mice27. Our results provide an explanation for increased oligoclonal T cell responses in the CNS that may, in part, derive from peripheral immune responses.

We show that peripherally induced T RM cells in the CNS exhibit dynamic movement behavior, resulting in an average speed of approximately 4 μm min−1. For comparison T RM cells in the skin, which are largely CD69−CD103+, are relatively slower (<2 μm min−1 average speed31) while T RM cells in the FRT, which are largely CD69+CD103+ (ref. 19), are relatively faster, averaging ~10 μm min−1 (ref. 19). Three main types of CD8+ T cell dynamic behaviors were observed in brains of peripherally infected mice: static/stationary, patrolling along vessels and seemingly random movement. A substantial proportion of CD8+ T cells were adjacent to blood vessels and these were primarily arrested. Of note, when administering anti-Thy1.1-PE i.v. before brain imaging of eGFP+ OT-I T cells, double-positive, likely perivascular cells were identified, which displayed reduced average speeds and increased arrest coefficients compared to eGFP single-positive (parenchymal) cells. Upon peripheral depletion, arrest coefficients in brain CD8+ T cells were reduced and average speeds increased, suggesting loss of cells that were mainly arrested (likely perivascular cells that were accessible to i.v. labeling). Since peripheral depletion did not compromise protection, it seems that the perivascular T cells were not major contributors to the early control of pathogens after i.c. infection.

Other brain CD8+ T cells showed patrolling movement along the vessel walls. Movement was not uniform in direction and these cells were often found reversing direction along the same vessel wall. Given the important role that LFA-1 plays in similar liver T RM cell patrolling patterns22 and the particularly high expression of CD11a on IV CD8+ T RM cells in the brain, it will be of interest to determine the role of adhesion molecules in brain T RM cell patrolling behavior. The last major dynamic behavior seen in peripherally induced T RM cells involved seemingly random movements. Brain T RM populations have high expression of CXCR6 and glial cells express CXCL16, the ligand for CXCR6 (ref. 50), but their role in T cell dynamics in the brain remains to be determined. Learning more about what drives the dynamic properties of T cells in the brain will provide valuable information that can be used to potentially alter behavior under pathogenic conditions such as neurodegeneration or after CNS infections.

Understanding how immune cells contribute to clearance of brain infections is critical to combat emerging pathogens including West Nile virus and Japanese encephalitis virus that can induce encephalitis and meningitis. Based on the notion that CNS infection may not be necessary for development of protective T RM cells in the brain, peripherally administered vaccines could potentially be used to elicit antigen-specific T cell response in the CNS in addition to the periphery to protect against some of these diseases. The ability to induce both peripheral and CNS resident memory cells could be beneficial to limit pathogen replication should peripheral immunity fail to completely inhibit pathogen entry into the CNS. Further studies will continue to shed light on the precise role of peripherally induced brain T RM cells.

The specificity of brain-residing T cells in humans remains largely unknown. A recent study identified oligoclonal CD8+ T cells patrolling the cerebrospinal fluid of patients with Alzheimer’s disease29 and at least some cells were specific for Epstein–Barr virus, an infection that originates in the periphery. Although more studies are needed, our results provide new perspectives on how CNS T RM cells may be generated and their potential contributions to controlling CNS infections and other neurological disorders.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41590-020-0711-8.

Received: 28 March 2019; Accepted: 13 May 2020; Published online: 22 June 2020

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Methods

Mice. The 6–10-week-old C57BL/6 and B6-Ly5.1/Cr mice were purchased from National Cancer Institute and NIH Swiss Webster mice were purchased from Envigo. Thy1.1 eGFP OT-I TCR-tg, Thy1.1 P14 TCR-tg, CD45.2 CD103KO P14 TCR-tg and CD103KO mice were bred in-house at the University of Iowa Animal Care Facility. The hC2D-Cre Runx3Lox (ref. 14) mice were crossed to P14 TCR-tg and were bred in-house at the University of Iowa Animal Care Facility. All animals were handled in accordance with guidelines established by the Institutional Animal Care and Use Committee.

Adoptive transfer. Thy1.1 eGFP OT-I or Thy1.1 P14 TCR-tg CDB8+ T cells were isolated from female donor blood and CD45.2 CD103KO P14 TCR-tg, hC2D-Cre Runx3Lox+ P14 TCR-tg and hC2DCre Runx3Lox+ P14 TCR-tg CDB8+ T cells were isolated from male donor blood. Red blood cells (RBs) were lysed using 1x Vitalyte (CytoMedica Group), frequencies of TCR-tg cells were determined by flow cytometry and approximately 0.5–2.5×10^9 TCR-tg cells were adoptively transferred via tail-vein injection into recipient mice 1 d before infection or immunization.

Infections and immunizations. DC prime boosting. DC priming and various boosting strategies were adapted from previous works. Briefly, lipopolysaccharide-matured EMS-like tyrosine kinase 3 ligand (Flt-3L)-induced splenic DCs digested with collagenase/DNase were incubated for 2 h at 37°C with 2μM OVA(257–264) or GP33(33–41) peptide. CD11c+ cells were enriched using anti-CD11c microbeads (Miltenyi Biotec) and ~5×10^6 peptide-coated DCs were i.v. injected into mice. Seven days later, mice were boosted i.v. with 1×10^5 c.f.u. of recombinant attenuated (ActA−) Listeria monocytogenes (Envigo. Thy1.1 eGFP OT-I TCR-tg, Thy1.1 P14 TCR-tg, CD45.2 CD103KO P14 TCR-tg and CD103KO mice were bred in-house at the University of Iowa Animal Care Facility. All animals were handled in accordance with guidelines established by the Institutional Animal Care and Use Committee.

Intravital two-photon microscopy. Intravital two-photon imaging was performed by Admera Health. Upregulated or downregulated genes in CD8−, eGFP or Thy1.1, CD103, CD11a, CXCR6, CD69 and CX3CR1. FSC, SSC, time, IV labeling and fixable viability stain were not used for clustering. IV labeling and fixable viability stain were excluded because populations were gated based on lack of expression by these markers. Additionally, Thy1.1 and eGFP were excluded from analysis when comparing Ig populations from different infectious conditions. SP analysis was run with a 200-node target and 10% downsampling.

RNA-seq and analysis. Total RNA was extracted from OT-1 cells that were isolated and sorted at a memory time point from the spleen, IV− and IV+ brains of DC-CAV-rLM-OVA immunized mice and two biological replicates, pooled from five mice each for brain samples were obtained for each group. RNA integrity was assessed using RNA 6000 Pico Kit for Bioanalyzer (Agilent Technologies, 5067-4503) and RNA-seq libraries were constructed using NEBNext Ultra RNA Library Prep Kit for Illumina (NEB, E6360) following manufacturer’s instructions. Briefly, between 1 and 10 ng of total RNA were converted to complementary DNA, utilizing RT primer and template switching oligo containing annealing sites for cDNA amplification primers. The cDNA was amplified with 14 cycles of PCR, enzymatically fragmented to an average size of ~250 bp, end-repaired, dA-tailed and ligated to NEBNext hairpin adaptors (NEB, E7335). After ligation, adaptors were converted to the ‘Y’ shape by treating with USER enzyme and DNA fragments were purified with 0.8x volume of Agencourt AMPure XP beads (Beckman Coulter, A63880). Adapter-ligated DNA was PCR amplified for eight cycles followed by AMPure XP bead cleanup. Libraries were quantified with Qubit dsDNA HS Kit (Thermo Fisher Scientific, Q2884) and the size distribution was confirmed with High Sensitivity DNA Kit for Bioanalyzer (Agilent Technologies, 5067-4626). Library quality and quantity were assessed with Qubit 2.0 DNA HS assay (Thermo Fisher Scientific), Tapestation High Sensitivity D1000 Assay (Agilent Technologies) and QuantStudio 5 System (Applied Biosystems). Equimolar pools of libraries was performed and sequenced on the HiSeq X Ten, with 2×150 bp paired-end reads. Reads were mapped onto Illumea Hiseq 2×150 bp format. Sequencing quality of RNA-seq libraries (assessed by FastQC v0.11.3), sequence alignment (determined using STAR v2.7.1a), gene expression (calculated with feature Counts v1.6.0) and differential gene expression analysis using DESeq2 (v1.22.2) initial analysis was performed by Admera Health. Upregulated or downregulated genes in CD8+, IV− versus IV+ were identified by requiring a fold change ≥2.5 and p value <0.05. Differentially expressed genes represented as heat maps were generated using GraphPad Prism.

Intravital two-photon microscopy. Intravital imaging of the CNS was performed using an adapted protocol15. In brief, mice were injected with intravascular dye (200μl of 0.1% Evans blue) and 10μg anti-Thy1.1 PE where noted before being anesthetized i.p. with avertin (400μl to 20g). Mice were monitored for movement pedal reflex (toe pinch) to ensure unresponsiveness and were monitored with a high-speed video camera throughout surgery and given additional anesthetic as needed. Ophthalmic ointment (artificial tears, Akorn Animal Health) was applied to both eyes to prevent corneal desiccation. The anesthetized mouse was positioned on a stage (5G-4N, head holder for mice, Narishige) where the ear bars and nose clamp were tightened to prevent movement of the head. Mouse temperature was maintained by use of a heating pad during surgery. Hair was removed from the top of the skull and the area was cleaned with 70% ethanol before incisions. Residual hair was removed from the skull via sterile q-tips. An outline of the window area (approximately 5 mm in diameter) lateral to the midline was performed and used light pressure the skull was slowly thinned to ~20–30 microns using a low-speed drill and burr bit (Fine Science Tools, 19008-09). A micro surgical blade (Surgistar, 6961) was used to smooth out areas as needed.

Mice were positioned in a temperature controlled environmental chamber at 37°C and were imaged using Leica SP8 Laser Scanning Microscope with Nonlinear Optics equipped with an 8000-Hz resonant scanner, a 25x motor-collared dipping objective (1.0 NA), an InSight DeepSee tunable laser (Spectra-Physics)
tuned to 940 nm (for eGFP, Evans blue, PE and secondary harmonic generation), a quad Hyd external detector array equipped with SP565 beam splitter detecting secondary harmonic generation (435–485 nm), eGFP (500–550 nm), Evans blue (665–705 nm) and PE (565–605 nm). Time-lapsed three-dimensional images were collected using a 2-μm z-step size with 20–40 s per stack for z-stack sizes of approximately 60–100 μm.

**Two-photon image analysis.** Three-dimensional time-lapsed z-stacks were imported into Imaris (v.9.1) software for analysis. The eGFP+ OT-I and Thy1.1+ OT-I cellular dynamics were determined using the spots function. For experiments with both eGFP and Thy1.1 PE, a co-localization channel was made for the double-positive cells. Cells were followed individually to ensure correct tracking and drift correction was used as needed. A surface object was created to form blood vessels. Cellular dynamics were determined to be inside or outside blood vessels based on whether they were masked by the vessel surface object. Average speed was calculated based on total track length. Arrest coefficient was calculated based on the proportion of time the cell was moving at less than 2 μm min⁻¹, with 1 being completely arrested and 0 indicating that the cell is always moving faster than 2 μm min⁻¹.

**Statistical analysis.** All statistical analyses were performed using GraphPad Prism (v.8.0). Where appropriate, two-tailed unpaired Student's t-tests or Mann–Whitney U-tests were performed comparing two groups and one-way ANOVA was used to compare more than two groups with Tukey's multiple comparisons test to identify significance between each group. Individual P values are provided in figure legends.

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**
The raw flow cytometric data that support the findings in SPADE analyses in Figs. 2, 3 and 5 are available from the corresponding author upon request. The RNA-seq data are deposited at the GEO with accession number GSE146077.

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**Acknowledgements**
We thank S. Perlman for critical review and comments on the manuscript and S. Anthony for helpful discussion. We thank J. Fishbaugh, H. Vignes and M. Shye (University of Iowa Flow Cytometry Core Facility) for cell sorting, I. Antoshechkin (California Institute of Technology) and Admera health for RNA-seq. Data herein were obtained from the Flow Cytometry Facility, which is a Carver College of Medicine Core Research Facilities/Holden Comprehensive Cancer Center Core Laboratory at the University of Iowa. This work was supported by grants from the National Institutes of Health (AI42767 to J.T.H., AI114543 to J.T.H. and V.P.B., GM134880 to V.P.B., AI121080 and AI139874 to H.-H.X., T32 AI007343 to S.L.U. and T32 AI007511 to I.J.J.) and the Veteran Affairs BLR&D Merit Review Program (BX002903) to H.-H.X.

**Author contributions**
S.L.U. and J.T.H. designed experiments; S.L.U. conducted experiments; S.L.U. and I.J.J. analyzed data; I.J.J., Q.S. and L.L.P. provided technical assistance; V.P.B. and H.-H.X. provided essential reagents and intellectual input and S.L.U. and J.T.H. wrote the manuscript.

**Competing interests**
The authors declare no competing interests.

**Additional information**
Extended data is available for this paper at https://doi.org/10.1038/s41590-020-0711-8.
Supplementary information is available for this paper at https://doi.org/10.1038/s41590-020-0711-8.
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Editor recognition statement L. A. Dempsey was the primary editor on this article and managed its editorial process and peer review in collaboration with the rest of the editorial team.
Extended Data Fig. 1 | Enrichment of antigen specific CD8+ T-cells in the CNS after peripheral immunizations. a, Gating strategy used to identify CD8+ T cell populations isolated from the CNS. Representative of 3 independent experiments with 4 mice per group. b–e, The proportion of donor OT-I, P14, or OVA tetramer+ cells of live CD8+ T-cells isolated from the Spleen (SP) peripheral blood (PBL) IV+ brain (IV+) or IV– brain (IV–) are graphed after infection with LCMV IV, p values (top to bottom) **** p < 0.0001, **** p < 0.0001, **** p = 0.0038, and * p = 0.0252 (b), DC-OVA-rLM-OVA prime boost IV, p values (top to bottom) **** p < 0.0001, **** p < 0.0001, ** p = 0.0002, *** p = 0.0004, and *** p = 0.0005 (c), DC-GP33-rLM-GP33 prime boost IV, p values (top to bottom) *** p = 0.0003, *** p = 0.0002, and **** p < 0.0001 (d), DC-OVA-VACV-OVA IV, p values (top to bottom) ** p = 0.0014, ** p = 0.0019, and * p = 0.0201 (e). Data represent from 2 independent experiments with 3 mice per group. Graphs show the mean +/- standard deviation with each dot representing an individual mouse. Statistical significance was determined by One-way ANOVA with Tukey’s multiple comparisons test across all the groups using graphpad prism. f, NIH Swiss Webster mice were infected with rLM-OVA IV and the proportion of live CD8+, IV– CD8+ and number of IV– CD8+ cells in the CNS are graphed. Data pooled from 2 independent experiments for a total of 10 mice per group. Graphs show the mean +/- standard deviation with each dot representing an individual mouse. Two-tailed unpaired students T-test were determined using graphpad prism and p values (left to right) * p = 0.0458, ** p = 0.0048, and * p = 0.0216.
Extended Data Fig. 2 | Runx3 but not CD103 are required for generation of peripherally induced CNS T rm cells. a, b, RNA-Seq analysis of memory OT-1 cells isolated from SP, IV+ or IV- brains of DC-OVA-rLM-OVA prime boosted mice. Fold change of IV+ vs IV- OT-1 cells of corresponding genes identified in Figure 2d (a). Fold change of IV+ vs IV- OT-1 cells of 40 different transcription factors associated with Trm or memory CD8+ T cell responses with selected genes indicated (b). RNA samples were isolated from 5 mice pooled per group in duplicate. c, CD103KO and Wt mice were DC-OVA-rLM-OVA prime-boosted and brains were harvested at a memory time point. The proportion and numbers of ova-specific CD69+ IV- CD8+ T-cells are plotted. Graphs show the mean +/- standard deviation with each dot representing an individual mouse. Data are representative of 2 individual experiments with 3 mice per group. d, e, Wt or CD103KO P14 cells from naïve donors were adoptively transferred into Wt CD45.1 hosts prior to LCMV infection IP. At a memory time point, proportion of donor P14 cells were determined and are depicted for Wt P14 cells (d) and CD103KO P14 cells (e). Graphs show the mean +/- standard deviation with each dot representing an individual mouse. Data are representative of 2 individual experiments with 5 mice per group. Statistical significance was determined by One-way ANOVA with Tukey’s multiple comparisons test across all the groups using graphpad prism with p values (top to bottom) **** p<0.0001, **** p<0.0001, and **** p<0.0001 (d) and ** p=0.0025, ** p=0.0026, and ** p=0.0033 (e). f, g, Wt or Runx3KO P14 cells were adoptively transferred into Wt CD45.1 hosts prior to LCMV infection IP. At a memory time point, proportion of donor P14 cells were determined and are depicted for Wt P14 cells (f) and Runx3KO P14 cells (g). Graphs show the mean +/- standard deviation with each dot representing an individual mouse. Data are representative of 2 individual experiments with 5 mice per group. Statistical significance was determined by One-way ANOVA with Tukey’s multiple comparisons test across all the groups using graphpad prism with p values (top to bottom) * p=0.0159, and * p=0.0279 (f).
Extended Data Fig. 3 | Increased representation of antigen specific CD8+ T-cells after peripheral immunizations is CNS specific. a-h, Thy1.1 eGFP+ OT-I T-cells were transferred into naïve mice that were DC-OVA-rLM-OVA prime boosted. Representative flow plots (a) showing proportion of OT-I cells of IV+ or IV- Thy1.1 CD8+ T-cells in each organ and cumulative proportion of OT-I cells in each organ, p values (left to right) ** p=0.0028 (b). Proportion of OT-I cells expressing CD103, p values (left to right) *** p=0.00371, * p=0.0100 (c) and CX3CR1, p values (left to right) *** p=0.0001, ** p=0.0027 (d) in IV+ vs IV- brain and meninges. gMFI of CD69, p values (left to right) *** p=0.0005, ** p=0.0001 (e), CXCR6, p values (left to right) **** p<0.0001, *** p=0.0004 (f), and CD49a, p values (left to right) **** p<0.0001, ** p=0.0025 (g) of OT-I cells from IV+ vs IV- brain and meninges are shown. SPADE analysis of OT-I cells from each organ are depicted (h). Markers used to distinguish populations include CD8α, CD11a, CD44, CD49a, CD69, CD103, CXCR6, and CX3CR1. Data are representative of 2 independent experiments with 3 mice per group. Graphs show the mean +/- standard deviation with each dot representing an individual mouse. Two-tailed unpaired t-students T-test were determined using graphpad prism.
Extended Data Fig. 4 | Peripherally induced CNS Trm cells are resistant to systemic depletion. a, b. Thy.1.1 eGFP+ OT-I T-cells were transferred into naïve mice that were DC-OVA-rLM-OVA prime boosted. At a memory time point, mice were control treated (PBS) or treated with 2, 5, or 10 μg of anti-Thy1.1 Ab IP. One week after depletion, proportions and phenotype of OT-I cells were determined. Representative flow plots gated on live CD8+ cells from Spleen, PBL and IV+ or IV− brain showing proportions of OT-I cells (a) and gMFI of CD69 gated on IV− OT-I cells (b). Graphs show the mean ± standard deviation with each dot representing an individual mouse. Data are representative of 2 individual experiments with 3 mice per group. Statistical significance was determined by One-way ANOVA with Tukey’s multiple comparisons test across all the groups using graphpad prism with p values (top to bottom) *** p=0.0005, *** p=0.0004, and * p=0.0144. c. Thy.1.1 eGFP+ OT-I T-cells were transferred into naïve mice that were DC-OVA-rLM-OVA prime boosted. After memory formation, mice were control treated (PBS) or treated with 2 μg of anti-Thy1.1 Ab IP. One week after depletion, OT-I cells in the CNS were imaged and maximum speed was determined. Graph depicts mean with each dot representing an individual OT-I cell from 71 cells from 12 individual movies (PBS), and 103 cells from 16 individual movies (2 μg). Statistical significance was determined by two-tailed Mann Whitney test using graphpad prism with **** p<0.0001.
Extended Data Fig. 5 | Enhanced recall response of peripherally induced brain Trm cells is specific to CNS. a, b, OT-I T-cells were transferred into recipient mice that were DC-OVA-VACV-OVA prime boosted. At a memory time point, mice were either unchallenged or challenged with rLM-OVA IC and organs were harvested 2 days later and stained for CD25 and intracellular IFN-γ directly ex vivo. Proportion of CD25+, p value **** p<0.0001 (a) and IFN-γ+, p value *** p=0.0004 (b) OT-I cells from each organ are graphed. Graphs show the mean +/- standard deviation with each dot representing an individual mouse. Data are representative of 2 independent experiments with 4 mice per group. Statistical significance was determined by two-tailed unpaired students t-test for each organ using graphpad prism.

c-e, P14 T-cells were transferred into recipient mice that were immunized with LCMV IP. At a memory time point, mice were treated with PBS or 2 μg anti-Thy1.1 Ab IP. One week post depletion, mice were challenged with rLM-GP33 IC and organs were harvested 2 days later and stained for CD25 and intracellular IFN-γ directly ex vivo. Proportion of CD25+, p values (left to right) ** p=0.0099, *** p=0.0014 (c), IFN-γ+, p values (left to right) * p=0.0308, ** p=0.0069 (d) and number of IV– P14 cells (e) from the brain are graphed. Graphs show the mean +/- standard deviation with each dot representing an individual mouse. Data are representative of 2 independent experiments with 3 mice per group. Two-tailed unpaired students T-test was determined using graphpad prism.
Extended Data Fig. 6 | See next page for caption.
Extended Data Fig. 6 | Peripherally induced brain resident CD8\(^+\) T-cells mediate protection against CNS infections. 

\(\textbf{a}\), Thy1.1 OT-I cells were transferred into mice and DC-OVA-rLM-OVA prime boosted. After memory formation, these mice and naïve controls were challenged with VSV-OVA IN. Virus titers in brains were determined at day 3 post infection. Data are combined from 2 independent experiments for a total of 10 mice per group. Graph shows the mean +/- standard deviation with each dot representing an individual mouse. Two-tailed unpaired students T-test was determined using graphpad prism where "" p=0.0021. 

\(\textbf{b}\), Thy1.1 OT-I cells were transferred into recipient mice that were DC-OVA-rLM-OVA prime boosted. After memory formation, mice were treated with PBS or 2 μg anti-Thy1.1 Ab IP. One week after depletion these mice and naïve controls mice were challenged with VSV-OVA IC. Kaplan Meier survival curves depicted. Data from representative experiment with 6 (black), 8 (red), and 9 (blue) mice per group (top to bottom). Graphpad prism used to determine significance using Mantel-Cox test for each group comparing to the Naïve + IC challenge group with p values * p=0.014 (red), and ** p=0.0056 (blue). 

\(\textbf{c, d}\), Thy1.1 OT-I or P14 cells were transferred into recipient mice that were DC-OVA-rLM-OVA or DC-GP33-rLM-GP33 prime boosted, respectively. After memory formation, mice were treated with PBS or 2 μg anti-Thy1.1 Ab IP. Frequency of OVA or GP33 tetramer positive cells and proportion of transgenic OT-I or P14 T-cells were determined prior to and 5 days post depletion. Representative flow plots (\(\textbf{c}\)) and cumulative data (\(\textbf{d}\)) of 2 independent experiments with 5 mice per group are shown.
Reporting Summary

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

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- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted. Give P values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings.
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes.
- Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated.

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection
- Flow Cytometry: BD LS98Fortessa, FacsDive software
- Intravital imaging: Leica SP8 two-photon microscope, LAS X software
- RNA-Seq: HiSeq 4000

Data analysis
- Flow Cytometry: FlowJo software version 9.9.4
- Image Analysis: Imaris version 9.1.0
- Statistical analysis: Graphpad Prism 8.1
- SPADE analysis: https://premium.cytobank.org/
- RNA-Seq: DESeq2 package (v 1.22.2)

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The raw flow cytometric data that support the findings in SPADE analyses in figures 2, 3 and 5, are available from corresponding author upon request.

RNA-Seq data have been deposited to the Gene Expression Omnibus (GEO) GSE146077

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data
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For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-list.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size
- Predetermined sample sizes were not determined. Sample sizes were determined based on prior observations and publications in addition to feasibility of experiments. For flow cytometric experiments, 3-5 animals per group were used and all experiments were repeated at least 2 times. For imaging studies, at least 3-5 mice were imaged using 2-5 fields per mouse. Sample sizes were determine based on previous studies using immunization strategies including LM, DC-LM, LCMV, IAV and VV.

Data exclusions
- Data were not excluded.

Replication
- All experiments were reproduced in at least 2 [if not more] independent experiments with biological replicates. The number of individual replicates can be found in the figure legends for each experiment.

Randomization
- 6-10 week old C57Bl/6 or Swiss Webster female mice were age-matched and randomly assigned to each group.

Blinding
- Investigators were not blinded during data analysis. Most experiments blinding was not necessary since comparisons were made within the same animal. For other experiments, blinding was not possible due to symptoms of infected mice.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

| Materials & experimental systems | Methods |
|----------------------------------|---------|
| n/a | n/a |
| Involved in the study | Involved in the study |
| Antibodies | ChIP-seq |
| Eukaryotic cell lines | Flow cytometry |
| Palaeontology | MRI-based neuroimaging |
| Animals and other organisms | | |
| Human research participants | | |
| Clinical data | | |

Antibodies

The following antibodies were obtained from BioLegend (BL), eBioscience (eBio), Tonbo, or BD and used at a 1:200 dilution unless otherwise noted:

- CD44 Alexa 700 (IM7, BL, 103026, lot B244378)
- CD44 redFluor 710 (IM7, Tonbo, 80-0441-U100, lot C04410500919803)
- CD25 PE (PC61.5, eBio, 12-0251-83, lot E01155-1633)
- CXC33 BV421 (CCX33-173, BL, 126522, lot B221423)
- CD11a BV510 (M17/4, BD, 563669, lot 9211589)
- CX3CR1 PECy7 (SA011F11, BL, 169016, lot B204072 B216575)
- CD69 PE-CF594 (H1.2F3, BD, 562455, lot 9024764 9214443)
- CD43 PECy7 (18B11, BL, 121218, lot B230797)
- CD103 PerCP-eFlour 710 (Z97, eBio, 46-1061-82, lot 4307224)
- CXC16 PE (SA051D1, BL, 151104, lot B223711 B269739)
- KLRG1 eFlour 450 (2F1, eBio, 48-5892-82, lot 4721587 4336605)
- CD127 PE (5B/199, BD, 121112, lot B189668)
- CD49a BV700 (Ha31/8, BD, 742164, lot 9198131 9339161)
- CD27 PECy7 (LG 3A10, BL, 124216, lot B211451)
- CD122 PE (SH4, eBio 12-1221-83, 1:100, lot E01459-1630)
- Thy1.1 FITC (OX-7, BL, 202504, 1:500, lot B137281 B206115)
- Thy1.1 PE (OX-7, BL, 202524, 10ug/mouse IV, lot B262497)
- CD62L eFluor 450 (MLT-14, eBio, 48-0621-82, lot 4336902)
- TNF PECy7 (MP6-XT22, BL, 506324, lot B251191)
Validation

All reagents are commercially available and each company has validated each antibody for application and species use. Further information regarding validation approaches can be found at:
- BioLegend (BL): https://www.biologend.com/en-us/reproducibility
- eBioscience / Invitrogen: https://www.thermofisher.com/us/en/home/life-science/antibodies/invitrogen-antibody-validation.html
- Tonbo: https://tonbobio.com/pages/validated-reagents-trusted-by-your-peers
- BD Bioscience: https://wwwbdbiosciences.ca/us/go/reproducibility/overview

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s) | Vero cell lines were originally obtained from American Type Culture Collection (ATCC) (Manassas, VA)

Authentication | Cell line purchased from ATCC was not authenticated

Mycoplasma contamination | Cell line was determined to be Mycoplasma free

Commonly misidentified lines (See ITAG register) | No misidentified cell lines were used in this study

Animals and other organisms

Policy information about studies involving animals, ARRIVE guidelines recommended for reporting animal research

Laboratory animals | 6-12 week old female C57Bl6/6 and male B6-Ly5.1/Cr mice were purchased from National Cancer Institute (Frederick, MD) and NIH Swiss Webster mice were purchased from Envigo (Indianapolis, IN). Thy1.1 eGFP OT-I T cell receptor (TCR) transgenic (tg), Thy1.1 P14 TCR tg CD45.2 CD103KO P14 TCR tg, and CD103KO mice were bred in house at the University of Iowa Animal Care Unit. hCD2-Cre+Runx3fl/Il were crossed to P14 TCR tg and were bred in house at the University of Iowa Animal Care Facility.

Wild animals | This study did not use wild animals

Field-collected samples | This study did not have field-collected samples

Ethics oversight | All animals were handled in accordance with guidelines established by the Institutional Animal Care and Use Committee under approved protocols at the University of Iowa

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots

Confirm that:
- The axis labels state the marker and fluorochrome used (e.g. CD4-FTC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation | Blood was collected 2min after IV injection just prior to harvesting organs including spleen, liver, kidneys, lungs and brain. Single cell suspensions of splenocytes were obtained by dissociating cells using frosted glass slides spleen followed by RBC lysis via ACK or Vidalys. Liver, kidney, and lungs were digested for 1 hour at 37°C with collagenase and DNase followed by dissociating tissue through 70μm filter. Cells were resuspended in 35% percoll (GE Healthcare, Chicago IL) and spun (1,500 RPM) at RT for 5min prior to RBC lysis via Vidalys. Brain mononuclear cells were isolated by digesting tissue in Collagenase/DNase for 45min at 37°C, dissociating through 70μm filter and isolating the interface between a 70% and 37% percoll gradient after a 20min RT spin at 2,000 RPM. Cells were enumerated and plated for staining.
| Instrument | Samples were acquired using an LSR Fortessa |
|------------|------------------------------------------|
| Software   | Data were analyzed using FlowJo software version 9.9.4 (Tree Star) |
| Cell population abundance | All CD8 T cells from each organ were gated on LiveDead^®^, FSC-W/FSC-A, and SSC-A/FSC-A to eliminate doublets, and histogram for CD8^+. IV^+ or IV^- CD8 T cells were gated and transgenic T cells were identified Thy1.1 expression. 1.5million SP OT-I cells, ~5800 and 6300 IV^+ brain OT-I cells, and 19000 and 29000 IV^- brain OT-I cells were sorted for low-abundance RNA sequencing. |
| Gating strategy | All CD8 T cells from each organ were gated on LiveDead^®^, FSC-W/FSC-A, and SSC-A/FSC-A to eliminate doublets, and histogram for CD8^+. IV^+ or IV^- CD8 T cells were gated and transgenic T cells were identified by eGFP or Thy1.1 expression. |

✓ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.