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Resident-memory CD8 T cells express high-affinity T cell receptors

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Abstract

Tissue-resident memory T (T_{RM}) cells serve as vanguards of anti-microbial host defense in nonlymphoid tissues, particularly at barrier epithelia and in organs with non-renewable cell types (e.g., brain). Here, we asked whether an augmented ability to sense antigen complemented their role as early alarms of pathogen invasion. Using mouse polyomavirus (MPyV), we show that brain-resident MPyV-specific CD8 T cells, unlike memory cells in the spleen, progressively increase binding to MHC class I tetramers and CD8 co-receptor expression. Using the two-dimensional micropipette adhesion frequency assay, we show that T_{RM} cells in brain, as well as in kidney, express up to 20-fold higher affinity T cell receptors (TCRs) than splenic memory T cells, whereas effector cells express TCRs of similar high affinity in all organs. Together, these data demonstrate that T_{RM} cells retain high TCR affinity, which endows them with the high antigen sensitivity needed for front-line defense against infectious agents.

Introduction

Anatomic location shapes the phenotypic and functional heterogeneity that defines subsets of memory T cells. Central memory T cells reside in secondary lymphoid organs, whereas effector memory T cells travel through the vasculature and enter nonlymphoid tissues. The third and largest subset of memory T cells does not recirculate and remains in fixed position in nonlymphoid tissues (1). These T_{RM} cells inhabit cutaneous and mucosal epithelia, portals of pathogen invasion where *in situ* initiation of immune defenses may prove essential for limiting host morbidity and mortality (2). These cells also occupy non-barrier sites (1, 3), of which the CNS may especially rely on T_{RM} cells to protect the large populations of non-regenerative cells. Brain CD8 T_{RM} cells have been characterized for acutely cleared CNS infections, such as vesicular stomatitis virus (VSV) and West Nile virus (4–6).

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JC polyomavirus (JCV) is an opportunistic pathogen in the human virome that can cause the life-threatening, demyelinating CNS disease progressive multifocal leukoencephalopathy (PML) under conditions of immunocompromise. Elevated frequencies of JCV-specific CD8 T cells correlate with improved PML prognosis in HIV/AIDS patients (7). Here, we identified high TCR affinity as a property of virus-specific CD8 T cells responding to persistent mouse polyomavirus (MPyV) infection in the brain. Using the two-dimensional micropipette adhesion frequency assay we discovered that anti-MPyV brain T_{RM} cells expressed TCRs having markedly higher affinity than virus-specific memory cells in the spleen. Virus-specific T_{RM} cells in the kidney, a major site of human polyomavirus persistence, also expressed high affinity TCRs. High TCR affinity would facilitate the ability of T_{RM} cells to sense viral antigens during low-level persistent infections.

Materials and Methods

Mice and Virus Inoculation

C57BL/6NCr female mice purchased from the Frederick Cancer Research and Development Center of the National Cancer Institute (Frederick, MD) were housed in accordance with the guidelines of the Institutional Animal Care and Use Committees of The Pennsylvania State University College of Medicine. Anesthetized mice (7–12 wk old) were intracerebrally (i.c.) injected in the right frontal lobe with 2×10^6 PFU MPyV strain A2 in 30 μl DMEM 5% FBS. Heat inactivated (70°C for 30 min) MPyV stock had no infectious virus by plaque assay (limit of detection 5 PFU/ml; not shown).

Quantitation of MPyV Genomes

TaqMan real-time PCR was performed with 10 μg of template DNA purified from tissue. Primers and amplification parameters were previously described (8).

mAb-mediated T Cell Depletion

Mice were injected i.p. with 250 μg of rat anti-CD8 (YTS169.4, BioXCell, West Lebanon, NH), rat anti-CD4 (GK1.5, BioXCell), or ChromPure whole rat IgG (Jackson ImmunoResearch, West Grove, PA) at 10 and 12 dpi, then weekly until 60 dpi. Lack of staining of PBMCs by anti-CD4 (RM4–5) or anti-CD8β (53–5.8) confirmed depletion.

Cell isolation, stimulation for intracellular cytokine staining, and flow cytometry

Brains, kidneys, and spleens were harvested from transcardially perfused mice. After collagenase digestion, brain and kidney cells were isolated on Percoll gradients, then exposed to Fixable Viability Dye (eBioscience, San Diego, CA) and Fc Block (BioLegend, San Diego, CA) prior to staining with D^b-LT359-368 tetramers (NIH Tetramer Core Facility, Atlanta, GA) and Abs to the following molecules: CD8α (53–6.7), CD4 (RM4–5), CD44 (IM7), CD69 (H1.2F3), CD103 (M290), CD62L (MEL14), IFN-γ (XMG1.2), and IL-2 (JES6-5H4) purchased from BD Biosciences (San Diego, CA); and TNF-α (TN3-19.12) purchased from eBioscience. BrdU (Sigma) was injected i.p. (1 mg/24 h) and mice euthanized 60 h later. BrdU uptake was detected using the BrdU Flow Kit (BD Biosciences). *Ex vivo* LT359 peptide stimulation and intracellular cytokine staining were

done as previously described (8). Samples were acquired on an LSR II or LSRFortessa (BD Biosciences) and data analyzed using FlowJo software (Tree Star, Ashland, OR).

Micropipette adhesion frequency assay

CD8 T cells were purified by magnetic sorting of mononuclear cells isolated from brain, kidney, and spleen. Coating human RBCs with pMHC, quantifying binding events and pMHC and TCR surface densities, and calculating adhesion frequency and 2-D affinity are as described (9, 10). A T cell that bound a D^b-LT359-coated RBC with an adhesion frequency of 0.1 or greater was considered antigen-reactive. No antigen-reactive binding events occurred with LCMV D^b-NP396-coated RBCs (Suppl. Fig. 2B & C).

Statistical analysis

p values were determined by unpaired Student's t-test or one-way ANOVA using GraphPad Prism software (La Jolla, CA). All *p* values ≤0.05 were considered significant.

Results and Discussion

Brain-infiltrating MPyV-specific CD8 T cells are stably maintained

CD8 T cells likely check progression of JCV-PML disease (7) and are important for control of systemic MPyV infection (11). To investigate CD8 T cell responses to MPyV CNS infection, mice were inoculated intracerebrally (i.c.), a route commonly used in neurotropic virus infection models (12, 13). To determine whether the brain supports MPyV replication, mice received infectious or heat-inactivated virus, resulting in a 100-fold increase in viral genomes from day 1 to day 4 post-infection (dpi) or no increase, respectively (data not shown). Viral genome numbers in the brain contracted approximately 100-fold from peak replication at 4 dpi to 8 dpi, followed by a low-level persistent infection phase (Fig. 1A); similar kinetics were observed in the spinal cord, kidney and spleen, as with intraperitoneal and subcutaneous inoculation routes. Next, we asked whether anti-viral T cell responses correlated with declining MPyV levels in the brain. Approximately half of brain-isolated cells at 8 dpi were CD8 T cells, of which nearly 75% bound D^b-LT359 tetramers (Fig. 1B), the immunodominant epitope (8). After peak infiltration at 8 dpi, both the magnitude of total CD8 T cells (Fig. 1C) and the frequency of D^b-LT359-specific CD8 T cells in the brain remained stable into the persistent phase, unlike the response in the spleen, which contracted approximately 30% (Fig. 1D). Most LT359-specific CD8 T cells in the brain produced IFN-γ at 30 dpi, with approximately 25% co-producing TNF-α (Fig. 1E). The CD4 T cell response in the brain mirrored that of CD8 T cells, although approximately 10-fold lower in magnitude throughout infection (Fig. 1C). These data show that brain-infiltrating CD8 T cells are predominantly directed to a single MPyV epitope, are functional, and are stably maintained in the setting of a nearly 3-log decrease in viral load.

MPyV-specific CD8 T cells in the brain are T_{RM} cells

We asked whether CD4 T cell help or continuous infiltration by circulating CD8 T cells in response to ongoing infection contributed to stability of this population. We depleted circulating cells by administering anti-CD4, anti-CD8, or control rat IgG at 10 dpi to allow peak infiltration of CD8 T cells into the brain, then weekly until 60 dpi. Total numbers of

brain-infiltrating CD4 T cells decreased in anti-CD4 treated mice, demonstrating their recruitment from the circulation during persistent infection (Fig. 2A). No change in numbers of total or MPyV-specific CD8 T cells in the brain were observed in CD4 or CD8 Ab-treated mice, indicating that neither CD4-help nor replenishment from the circulation is needed to maintain this population (Fig. 2A). This finding parallels parabiosis experiments in which endogenous memory cells in the brain do not equilibrate with donor parabiont cells, suggesting that effector-phase T cells seed the memory population (14). Next, we administered the thymidine analog BrdU to mice 60 h prior to sacrifice at 30 dpi. Approximately 15% of D^b-LT359-specific CD8 T cells in both the brain and spleen incorporated BrdU (Fig. 2B), suggesting that *in situ* proliferation contributed to the long-term maintenance of brain-infiltrating memory CD8 T cells.

We asked whether the canonical CD62L-selectin^{lo} CD69^{hi} CD103^{+} phenotype used to define T_{RM} cells in acute infection models applied to brain-infiltrating virus-specific CD8 T cells during persistent MPyV infection. Over 90% of D^b-LT359-specific CD8 T cells in the brain upregulated CD69 by 15 dpi, but those in the spleen remained CD69$^{-/lo}$ at all timepoints examined (Fig. 2C). All MPyV-specific CD8 T cells in the brain and spleen were CD62L^{lo}, a phenotype expected in persistently infected hosts. Notably, most MPyV-specific CD8 T cells in the brain remain CD69^{hi} despite declining virus levels (Fig. 1A). Because CD69 antagonizes expression of S1P1 receptors, which must be downregulated for establishment of a T_{RM} compartment (15), CD69^{hi} appears to be an indispensable phenotype for T_{RM} cells. Beginning at 15 dpi, we observed a gradual increase in CD103 expression by D^b-LT359-specific CD8 T cells; however, even at 30 dpi only one-third of these cells were CD103^{+} (Fig. 2C) with no further increase in CD103 expression by 60 dpi (data not shown). This contrasts with acutely cleared VSV infection in the CNS, in which the CD103^{+} fraction increases from approximately 60% to 80% of VSV-specific CD8 T cells from 20 to 40 dpi (4). Most MPyV-specific CD8 T cells in the brain were CD103^{-} despite being stably maintained, indicating that these cells were functionally brain-resident during persistent infection. Together, these data suggest that persistent antigen impedes CD103 upregulation, in line with other studies (16), but not establishment of a T_{RM} population. Evidence that CD103 expression by CD69^{hi} CD62L^{lo} CD8 T cells varies between nonlymphoid organs (15) further supports the likelihood that CD103 is not a reliable T_{RM} marker. The role of cytokines and antigen in development and retention of T_{RM} cells to low-level persistent infections remains to be determined. The gradual decline in virus levels during persistent infection and stability of MPyV-specific CD8 T cells imply that antigen may be dispensable for retention of these T_{RM} cells as for skin-resident HSV-specific T_{RM} cells (17).

Brain- and kidney-infiltrating memory MPyV-specific CD8 T cells express high affinity TCRs

MPyV-specific CD8 T cells in the brain showed a progressive increase in mean fluorescence intensity (MFI) of D^b-LT359 tetramer while in the spleen, tetramer MFI stayed uniformly lower during persistent infection (Fig. 3A). We also observed that brain-infiltrating cells expressed significantly higher levels of CD8 co-receptors than those in the spleen as early as 8 dpi and showed a progressive increase in MFI (Fig. 3B). The cooperative trimolecular interactions between the TCR, CD8, and pMHC complex obviate determination of affinity

of a given TCR for its cognate pMHC ligands (18). Therefore, to ask whether MPyV-specific memory T cells in the spleen and brain differed in TCR affinity, we used the micropipette adhesion frequency assay with D^b-LT359 monomers where the native α3 domain is replaced with that of HLA-A2, which cannot bind mouse CD8 (9). This assay allows interrogation of the affinity of a single T cell for pMHC in a physiologically relevant membrane-anchored context, and can detect antigen-specific populations with affinities below detection by tetramers (10, 19, 20). Reactivity to D^b-LT359 was assessed for 56 CD8 T cells isolated from the brain, 44 of which were determined to be antigen-specific (78.6%) (Fig. 4A). In contrast, only 14 CD8 T cells isolated from the spleen were found to be D^b-LT359-specific out of 68 cells interrogated (20.6%). The micropipette adhesion frequency data confirm the observations made with tetramers that D^b-LT359-specific CD8 T cells predominantly comprise the brain T_{RM} pool.

Using the adhesion frequency values together with TCR and pMHC surface densities, we calculated that brain T_{RM} cells have a 20-fold higher mean affinity for D^b-LT359 than cells in the spleen (Fig. 4B). To address whether increased TCR affinity was unique to T_{RM} in the brain, we assayed 69 kidney-infiltrating memory CD8 T cells and found that 55.1% were D^b-LT359-specific, with a mean TCR affinity significantly higher than those measured in the spleen ($p = 0.0029$). Cells in the brain and kidney occupied a 3-log range skewed toward higher affinities, in contrast to the spleen where affinities spanned 1-log (Fig. 4C). Notably, cells in the kidney did not differ significantly in affinity from those in the brain (Fig. 4B) and had higher MFIs for D^b-LT359 tetramer and CD8 Ab staining than those in the spleen (Suppl. Fig. 1A). Approximately 70% of CD8 T cells in the kidney expressed CD69, suggesting that most are T_{RM} cells (Suppl. Fig. 1B). Elevated CD8 levels of T cells in both brain and kidney may constitute an additional phenotypic marker for T_{RM} cells. Together, these data show that MPyV-specific CD8 T_{RM} cells express higher affinity TCRs with cooperative binding by higher levels of CD8 than memory cells in the spleen.

A number of studies document that effector, but not memory, CD8 T cells are competent to enter nonlymphoid tissues (4, 21, 22). Effector CD8 T cells generated *in vitro* express higher effective TCR affinity than their naïve precursors (9). Together, these findings raise the possibility that effector T cells in both lymphoid and nonlymphoid tissues may express high affinity TCRs during acute infection. We compared the TCR affinities of virus-specific CD8 T cells in acutely infected mice. From the brain, TCR affinities for D^b-LT359 were determined for 40 out of 53 cells assayed, a frequency of 75.4% (Suppl. Fig. 2A). In both the spleen and the kidney, the frequency of D^b-LT359-specfic CD8 T cells was lower, with only 40 out of 86 cells (46.5%) cells in the spleen and 15 of 33 (45.4%) cells from the kidney (Suppl. Fig. 2A). Unlike memory cells, effector T cells in all three organs expressed TCRs of similarly high affinity (geometric mean affinities: 7.72×10^{-4}, 5.20×10^{-4}, and 7.48×10^{-4} um^4 for the brain, kidney, and spleen, respectively) (Fig. 4D). For both acute and persistent infection time points, splenic D^b-LT359-specific CD8 T cells had significantly higher TCR surface density than those from the brain (Suppl. Fig. 2D & E), and this difference is taken into account for calculating TCR affinity (10). Similar to memory cells in the brain and kidney, CD8 T cells from all organs isolated during acute infection spanned a 3-log range of affinities.

These findings suggest that high-affinity T_{RM} cells originate from high-affinity effector cells that enter nonlymphoid tissues during acute infection; further, these data raise the possibility that the nonlymphoid microenvironment may be conducive for retaining T cells having high affinity TCRs. High TCR affinity would improve the ability of T_{RM} cells to detect infected cells expressing low levels of antigen, not only during persistent polyomavirus infection, but also during early re-infection when rapid control may be critical in limiting injury to organs with a large population of essential, non-renewable cells (e.g., brain).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used in this article

dpi	days postinfection
i.c.	intracerebral
(g)MFI	(geometric) mean fluorescence intensity
JCV	JC polyomavirus
MPyV	mouse polyomavirus
PML	progressive multifocal leukoencephalopathy
T_{RM}	tissue-resident memory T cell

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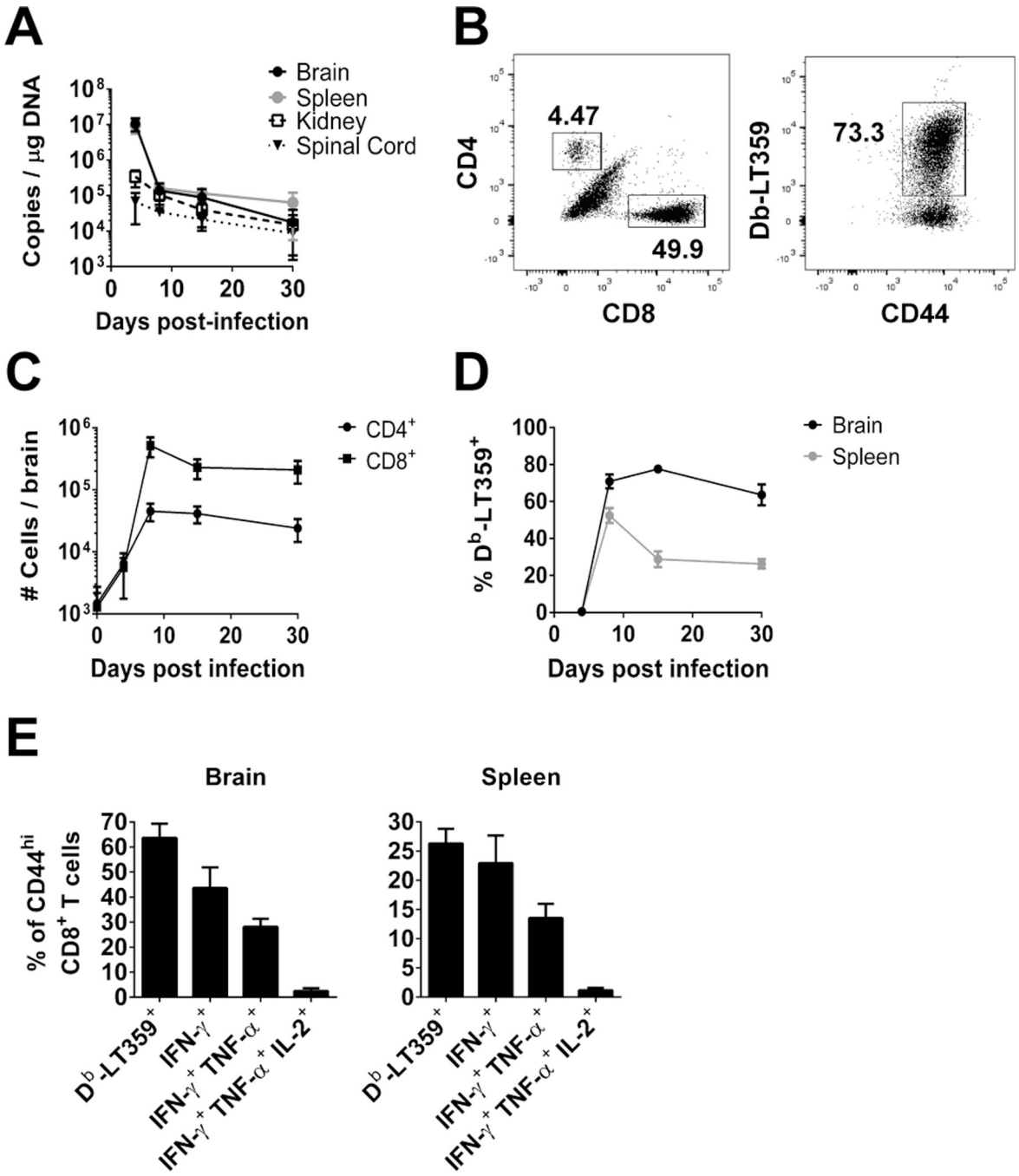

Fig. 1.

Characterization of MPyV-specific T cell responses in the brain. (A) Kinetic analysis by qPCR of viral genome copy numbers in genomic DNA isolated from brain, spleen, kidney, and spinal cord. (B) Representative dot plot of T cell frequencies in brain-isolated mononuclear cells (left plot) and frequency of D^b-LT359-tetramer$^+$ cells of total CD8 T cells (right plot) at 8 dpi by flow cytometry. (C) Kinetic analysis of the number of total CD8 and CD4 T cell responses in brain. (D) Frequency of D^b-LT359$^+$ of CD44^{hi} CD8$^+$ T cells in brain and spleen over the course of infection. (E) Frequency of cytokine-producing CD44^{hi}

CD8 T cells in brain and spleen at 30 dpi following *ex vivo* stimulation with LT359 peptide. Data are cumulative from 3 independent experiments with 7–9 total mice/timepoint.

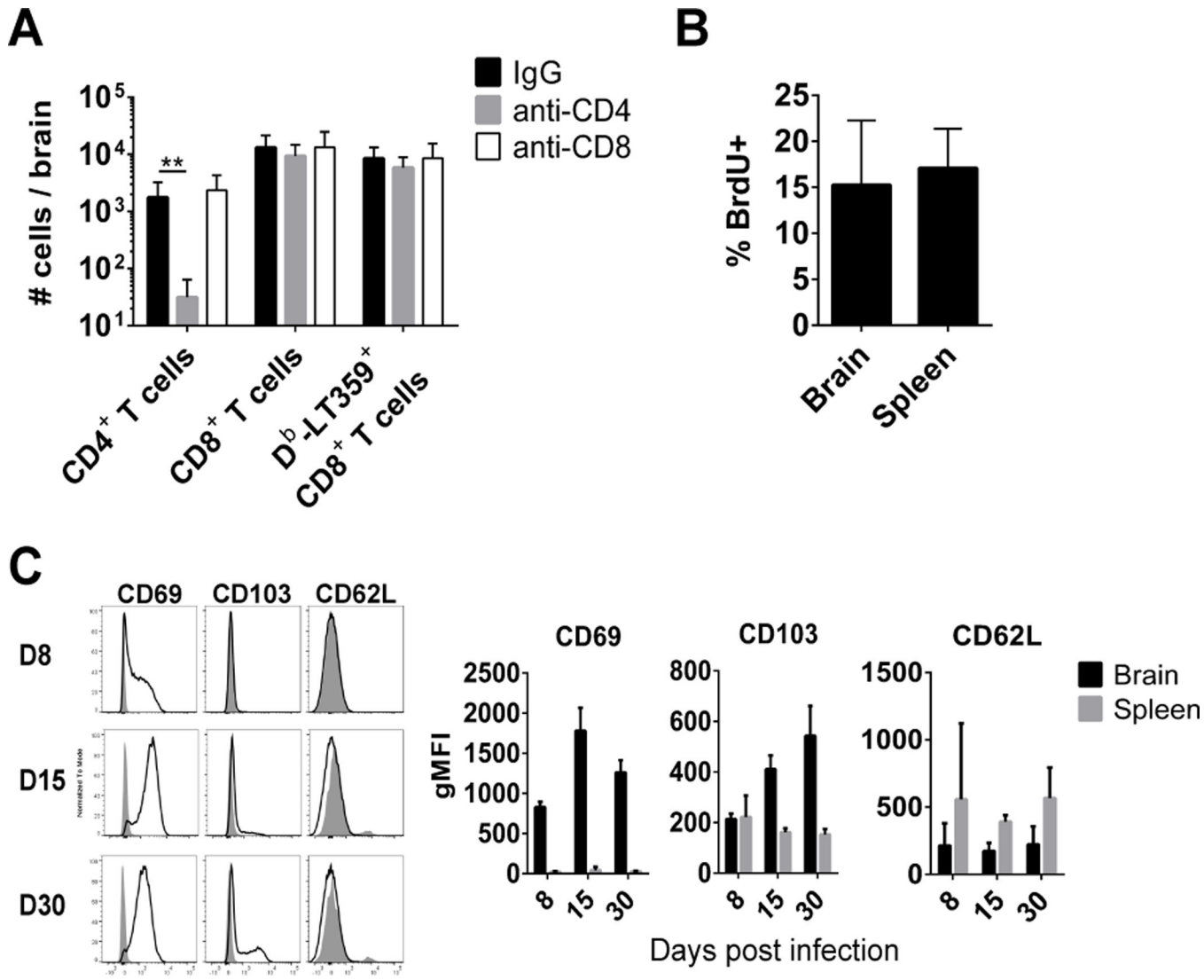

Fig. 2.

MPyV-specific CD8 T cells survive long-term in the brain. (A) Mice were Ab-depleted of circulating CD8 or CD4 T cells, and numbers of T cells infiltrating the brain were determined by flow cytometry. Data are cumulative from 2 independent experiments with 7–8 total mice/group. (B) Mice were given BrdU i.p. for 60 h prior to sacrifice at 30 dpi. Shown are the mean frequencies ± SD of $BrdU^+$ D^b-$LT359^+$ CD8 T cells in brain and spleen. Data are cumulative from 2 independent experiments with 9 total mice. (C) Representative histograms (left panel) of expression of phenotypic markers of tissue-resident memory cells by brain-infiltrating (line) or splenic (shaded) D^b-$LT359^+$ CD8 T cells at indicated timepoints post-infection. Right panel, mean ± SD of the gMFI of each marker; brain (black) and spleen (gray). Data are cumulative from 3 independent experiments of 7–9 mice/timepoint.

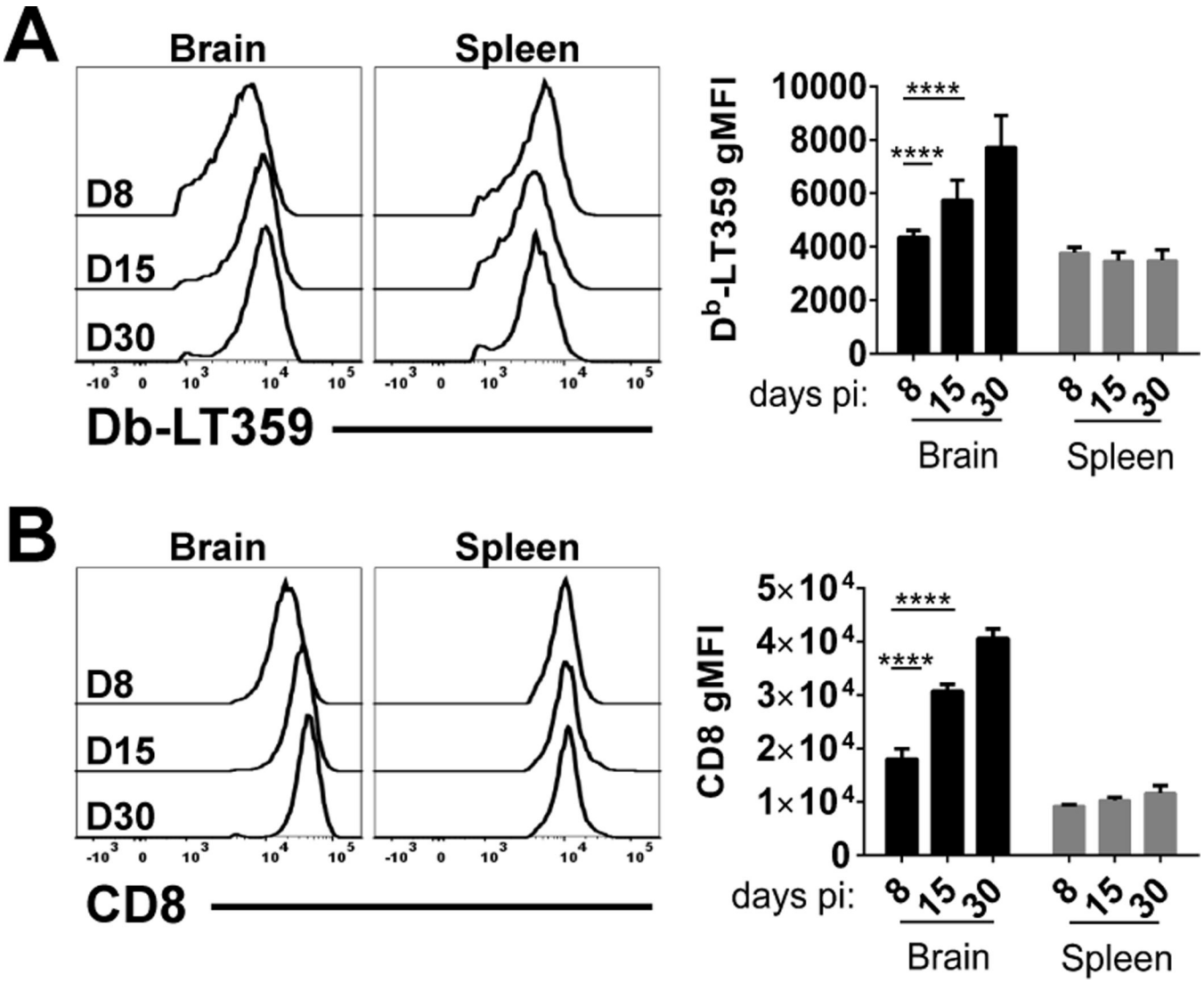

Fig. 3.

Increase in tetramer binding and CD8 expression by MPyV-specific CD8 T cells in the brain. CD8 T cells from brain and spleen were analyzed for geometric (g)MFI of staining by (A) D^b-LT359 tetramers and (B) anti-CD8α, shown by representative histograms (left panel) and mean ± SD (right panel). Data are cumulative from 3 independent experiments of 7–9 mice/timepoint. ****, $p < 0.0001$.

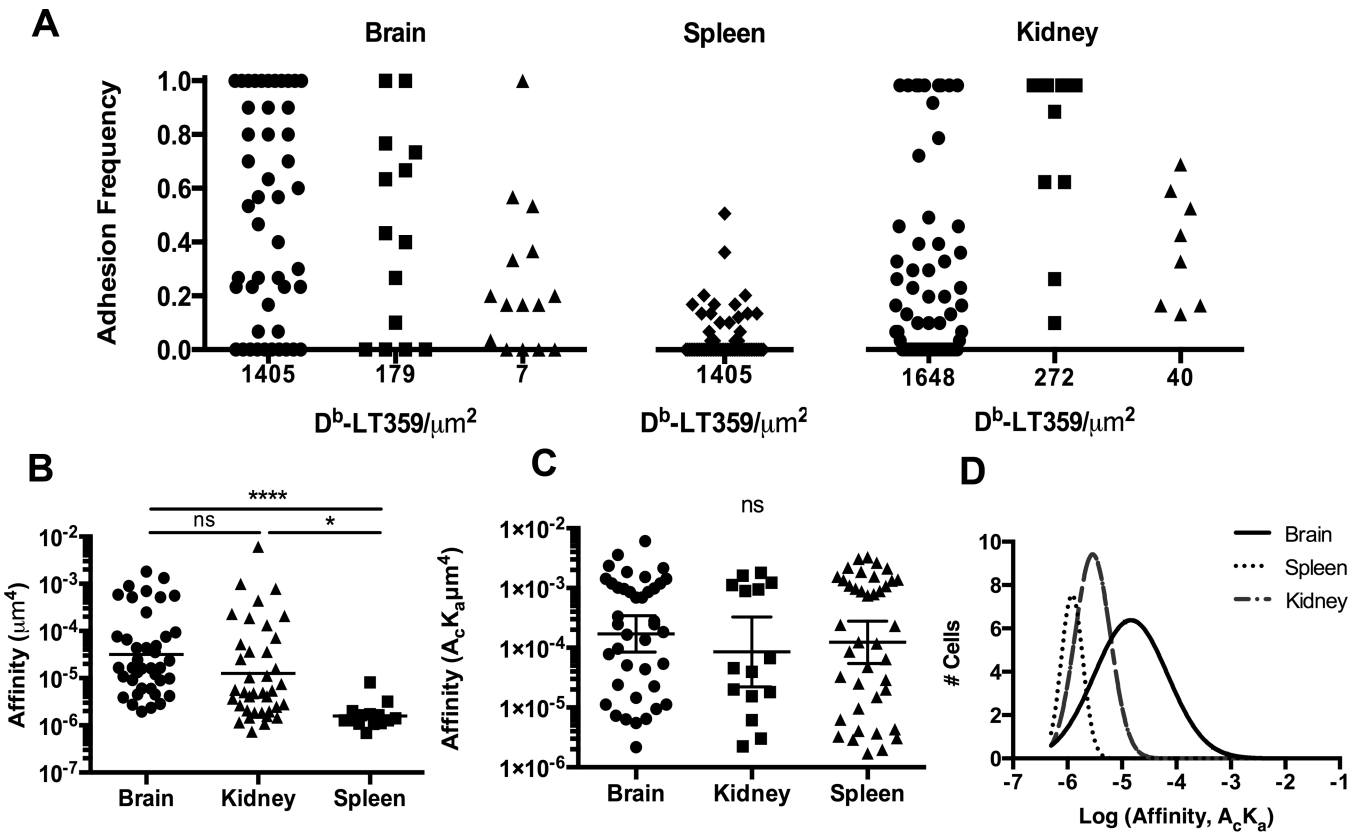

Fig. 4.

Comparison of TCR affinities of MPyV-specific CD8 T cells in brain, kidney, and spleen. CD8 T cells pooled from each organ during persistent (A–C; 26–32 dpi) or acute (D; 8 dpi) infection were analyzed by micropipette adhesion frequency assay. (A) Adhesion frequencies of CD8 T cells from the brain, spleen, and kidney using RBCs coated with different pMHC surface densities. (B & C) Geometric mean ± 95% confidence interval of 2-dimensional binding affinity for D^b-LT359, where each dot represents an individual CD8 T cell. (D) Frequency distributions of the log of affinities for each organ examined. Gaussian curves were fitted to the data with r^2 values as follows: spleen = 0.97; kidney = 0.71; brain = 0.64. Data are cumulative from 2 independent experiments with 5–7 mice/pool. ns = not significant; *, $p < 0.01$; ****, $p < 0.0001$.