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CD8⁺ T Cells Primed in the Periphery Provide Time-Bound Immune-Surveillance to the Central Nervous System

Kevin G. Young,*† Susanne MacLean,*† Renu Dudani,* Lakshmi Krishnan,*† and Subash Sad*†

After vaccination, memory CD8⁺ T cells migrate to different organs to mediate immune surveillance. In most nonlymphoid organs, following an infection, CD8⁺ T cells differentiate to become long-lived effector-memory cells, thereby providing long-term protection against a secondary infection. In this study, we demonstrated that Ag-specific CD8⁺ T cells that migrate to the mouse brain following a systemic Listeria infection do not display markers reminiscent of long-term memory cells. In contrast to spleen and other nonlymphoid organs, none of the CD8⁺ T cells in the brain reverted to a memory phenotype, and all of the cells were gradually eliminated. These nonmemory phenotype CD8⁺ T cells were found primarily within the choroid plexus, as well as in the cerebrospinal fluid-filled spaces. Entry of these CD8⁺ T cells into the brain was governed primarily by CD49d/VCAM-1, with the majority of entry occurring in the first week postinfection. When CD8⁺ T cells were injected directly into the brain parenchyma, cells that remained in the brain retained a highly activated (CD69hi) phenotype and were gradually lost, whereas those that migrated out to the spleen were CD69low and persisted long-term. These results revealed a mechanism of time-bound immune surveillance to the brain by CD8⁺ T cells that do not reside in the parenchyma. The Journal of Immunology, 2011, 187: 1192–1200.

Listeria monocytogenes is an intracellular pathogen that causes disease in immunocompromised hosts. The most severe outcomes in persons infected by L. monocytogenes occur when the bacteria invade the CNS, which can result in death (1). During primary infection, L. monocytogenes is controlled mainly by innate immunity (2, 3). In the brain, a primary infection with L. monocytogenes is controlled mainly by CD8⁺ T cells and NK cells, which develop after several days (1). Protection against a peripheral secondary infection is mediated mainly by CD8⁺ T cells through a mechanism that is IFN-γ independent (4) but perforin (5) and TNF (6) dependent.

CD8⁺ T cells are stimulated by APCs, which display peptides from endogenously derived Ags (intracellular bacteria, viruses, or tumors) on MHC class I molecules (7). After differentiation, CD8⁺ T cells secrete cytokines and mediate specific cytotoxicity (by perforin- and Fas-dependent pathways) toward infected cells and tumors (8–10). A vast majority (>95%) of Ag-specific T cells activated at the onset of the immune response are eliminated, and only a small portion of those T cells survive (<5%) for extended periods (11–13). These long-lived memory T cells possess the unique ability to respond rapidly and specifically to Ags (14, 15).

After activation of CD8⁺ T cells, the expression of various cell surface molecules is differentially modulated (15–17); however, CD44 is one of the few proteins that is persistently elevated on memory T cells, irrespective of their activation status (14, 18). Memory T cells have been segregated broadly into two phenotypic and functional subsets: effector memory cells (CD44hiCD62Llow CCR7⁻) and central memory cells (CD44hiCD62LhiCCR7⁺) (19–23). Central memory cells persist within the lymphoid organs, whereas effector memory cells home to nonlymphoid organs (24). Although both subsets can express cytokines (25), central memory cells can proliferate more efficiently and, consequently, provide better long-term protection (26).

T cells, after activation, traffic to various nonlymphoid organs to provide rapid protection against reinfection (24, 27–29). Although the blood–brain barrier (BBB) exists to prevent the exposure of the brain to various soluble mediators from systemic sites, multiple mechanisms of T cell trafficking to the brain have been proposed (30, 31). In this study, we evaluated the trafficking of Ag-specific CD8⁺ T cells to the brain during systemic infection of mice with the intracellular pathogen L. monocytogenes. Our results indicated that CD8⁺ T cells that reside in the brain display cell surface markers typical of early effectors and are found mainly outside of the parenchyma. Following a secondary brain infection, these cells move through cerebrospinal fluid (CSF)-filled spaces and into the parenchymal region affected by the infection. In the absence of a brain infection, these cells never revert to a memory phenotype and are gradually eliminated, thereby providing time-bound immune surveillance to the brain. Activated CD8⁺ T cells transferred directly into the brain parenchyma undergo gradual and complete erosion within the brain, but they can survive and convert to memory cells in the periphery following migration out of the brain. Thus, in the absence of Ag in the brain, CD8⁺ T cells may not convert to memory cells within this organ.
Materials and Methods

Bacterial strains

OVA-expressing *L. monocytogenes* (LM-OVA), as described previously (32), was grown in brain–heart infusion (BHI) media (DIFCO Laboratories, Detroit MI) to OD$_{600nm}$ = 0.4–0.8. At midlog phase, bacteria were harvested and frozen in 20% glycerol and stored at −80°C. CFU were determined by performing serial dilutions on plates.

Mice and immunizations

Female C57BL/6j mice were obtained at 6–8 wk of age from The Jackson Laboratories (Bar Harbor, ME). CD45.1+ OT-1 and CD45.2+ OT-1 mice were bred in-house. Mice were maintained in the animal facility at the National Research Council of Canada-Institute for Biological Sciences, in accordance with the guidelines of the Canadian Council on Animal Care. For immunizations with LM-OVA, frozen stocks were thawed and diluted in 0.9% NaCl, and mice were inoculated i.v. via the lateral tail vein with 1 × 10^6 LM-OVA suspended in 200 μl 0.9% NaCl.

For intracranial (i.c.) injections, mice were deeply anesthetized with isoflurane in O$_2$. The scalp was cut to expose the skull, and a 30-gauge burr hole was made through the skull above the left anterior forebrain. Intracranial injections of 1 × 10^1–10^6 LM-OVA in 5 μl saline were performed using a Hamilton syringe fitted with a 31-gauge blunt-end cannula. The cannula was left in situ for an additional 1 min to prevent reflux along the injection tract. A plug of sterile surgical bone wax was used to cover the hole in the skull, and mice were given bupivacaine (long-acting local anesthetic) before the skin was sutured and the sutures tied were glued.

Assessment of bacterial burden

Spleens and brains from infected mice were homogenized in RPMI 1640 medium. CFU were determined by plating 100-μl aliquots of serial 10-fold dilutions in 0.9% saline on BHI agar plates.

Isolation of cells from spleen, brain, lungs, and liver

A transcardial perfusion was performed in mice before isolation of organs. Mice were deeply anesthetized with isoflurane in O$_2$. The right atrial chamber of the heart was lacerated with scissors. A 25-gauge needle attached to a heparinized cannula was inserted into the left ventricular chamber. Mice were perfused with 50–60 ml PBS plus 1 ml 60% Percoll (GE Healthcare) and then layered onto 70% Percoll. Cells were resuspended in 1 ml RPMI 1640. The tissue was then pushed through a 100-μm cell strainer (Falcon 2360 cell strainer), using a plunger from a 10-ml syringe. The remaining cells were plotted for CD8 and OVA257–264-tetramer fluorescence using forward- versus side-scatter plots to separate them from glial and neuronal cells, and the remaining cells were plotted for CD8 and OVA237–264-tetramer fixed in 0.5% formaldehyde, and acquired on a BD FACSCanto flow cytometer. In some experiments, CD45.1+ OT-1 cells were adoptively transferred into CD45.2+ recipients, and the transferred cells were tracked after staining cells with anti-CD45.1 Abs.

Functional Abs

Blocking Abs against CD49d (9C10 and R1-2; BD Biosciences) and VCAM-1 (clone 429; eBioscience) were injected i.p. (100 μg/mouse). A rat isotype control, IgG2a × (eBioscience), was used in control injections compared with the VCAM-1–blocking Ab, and normal rat IgG was used as a control for CD49d blocking. The number of injections and their timing are detailed in the appropriate figure legends. The CD8 cell-depletion Ab (clone 2.43) was produced in our laboratory by a standard Protein G-purification protocol from hybridoma supernatant. It was diazylated against PBS, and its functional concentration was determined by an ELISA assay.

Imaging

A standard immunofluorescence-staining protocol was used for labeling 10-μm frozen sections cut from unfixed, PBS-perfused brains. The sections were fixed with 4% paraformaldehyde for 15–20 min, washed three times for 10 min in PBS, and then blocked and permeabilized with PBS containing 0.4% Triton X-100 and 10% donkey serum. Following the blocking/permeabilization of the sections, they were sequentially incubated with the different primary and secondary Abs, with three 10-min washes with PBS in between Ab incubations. Abs were diluted in a solution of PBS containing 0.04% Triton X-100 and 5% PBS and incubated either overnight at 4°C or for 1 h at room temperature. Primary Abs included goat anti-IL-7Rα (eBioscience), rabbit anti–IL-2Rα (BD Biosciences) at 1:100, rat anti-CD8α (clone 53-6.7; BD Biosciences) used at 1:200, mouse anti-CD45.1 (clone A20; BD Biosciences) at 1:200, rabbit anti-smooth muscle actin (Abcam) used at 1:200, and rabbit anti-pan cyto-keratin (Santa Cruz, H-240) used at 1:50. Secondary Abs were donkey anti-goat or donkey anti-rabbit Alexa Fluor 488 (Invitrogen), highly cross-adsorbed donkey anti-rat Cy3 (Jackson Immunoresearch), donkey anti-mouse Alexa Fluor 546 (Invitrogen), and highly cross-adsorbed donkey anti-rabbit Cy5 (Jackson Immunoresearch). Hoechst dye was used to label cell nuclei by diluting a 1 mg/ml solution 1:5000 in PBS and incubating it for 15 min on the sections, prior to a final wash and mounting in Dako fluorescent mounting medium. Images were acquired on a Zeiss Axiovert 200M microscope with an AxioCamHR3 camera, using appropriate filter cubes. Extended-focus images were generated by acquiring Z-series (at the optimal step size to satisfy the Nyquist frequency) using either a 20× (NA = 0.5) or 40× (NA = 0.75) Neofluor objective. Images were subsequently processed in Axiovision 4.7 (Zeiss), and tiff images were assembled with Adobe Photoshop and Adobe Illustrator (Adobe Systems) for the figures. The picture of the brain during cryosectioning was taken using an iPhone3 GS (Apple).

Results

Migration of effector phenotype CD8+ T cells to the brain following systemic LM-OVA infection

It was shown that *L. monocytogenes* (10403S), when given i.v. to mice, results in an acute infection that lasts until day 5–7 (32). We determined whether this strain of *L. monocytogenes* would also reach the brain of infected mice with the injection of 10^5 bacteria. Dilutions of the entire brain homogenate were plated on agar plates to detect bacterial colonies. As shown in Fig. 1A, LM-OVA was detectable in the spleens, but not the brain, of infected mice. By day 10, LM-OVA was undetectable in the spleens of infected mice. Not a single colony of LM-OVA was detectable in the brains of infected mice at any of the time intervals.

We then determined the relative migration of CD8^+ T cells to the brain in LM-OVA–infected mice that had been adoptively transferred with low numbers of OVA-responsive OT-1 cells prior to infection. Mice were perfused first with 50–100 ml of buffer, and the spleen and brain were removed. This was done to remove traces of blood from brain vasculature. Lymphocytes in the brain were detected by flow cytometry using forward- versus side-scatter plots to separate them from glial and neuronal cells, and the remaining cells were plotted for CD8 and OVA237–264 tetramer.
OV A-tetramer+ CD8+ T cells was evaluated in the brain and spleen. Each IL-7R Abs and analyzed by flow cytometry. Furthermore, OV A-specific CD8+ T cells in the brain expressed were also stained with Abs against CD62L (Fig. 1C). This was followed by a stable pool of memory CD8+ T cells. The numbers of OVA-tetramer+ CD8+ T cells in the brain and spleen were evaluated. D, To compare the rates of attrition, the percentage of cells remaining past day 5 postinfection were calculated, with numbers at the day 7 time point set as 100%. Cells were also stained with Abs against CD62L (E), CD11a (F), CD69 (G), IL-7Rα (H), and IL-2Rα (I), and the expression of these molecules on OVA-tetramer+ CD8+ T cells was evaluated in the brain and spleen. Each experiment involved the analysis of at least four mice/group and was repeated at least twice.

CD8+ T cells respond to fight a secondary infection of LM-OVA in the brain

CD8+ T cells are known to be a major player in the response to a primary brain infection of L. monocytogenes (1). To assess the importance of the CD8+ T cell response in the brain in fighting a potential secondary infection, we examined the response of mice to a brain rechallenge of LM-OVA following CD8+ T cell depletion. We first examined the survival of LM-OVA-immunized animals depleted of CD8+ T cells using an anti-CD8 depletion Ab (clone 2.43) and then given an i.c. rechallenge of LM-OVA (1 × 10^5). A second injection of the anti-CD8 depletion Ab was given following the i.c. infection. Depletion of OVA-specific CD8+ T cells with this Ab was complete by 24 h postinjection in brain staining (Fig. 1B). There was a rapid increase in the numbers of OVA-specific CD8+ T cells in the spleen that peaked at day 7 of LM-OVA infection and subsequently underwent attrition (Fig. 1C). This was followed by a stable pool of memory CD8+ T cells. The numbers of OVA-specific CD8+ T cells in the brain did not peak at day 7, and the numbers increased steadily until day 15, which was indicative of a progressive migration of cells to the brain (Fig. 1C). In the long term, the relative numbers of OVA-specific CD8+ T cells in the brain declined in a similar fashion to those in the spleen (Fig. 1D). This resulted in a loss of OVA-specific CD8+ T cells in the brain down to levels close to the detection limit, whereas a stable pool of memory cells remained in the spleen (Fig. 1D). Interestingly, OVA-specific CD8+ T cells in the brain were persistently CD62L^hi^CD11a^hi^ (Fig. 1E, 1F). Furthermore, OVA-specific CD8+ T cells in the brain expressed high levels of the early activation marker, CD69, even at day 180 postinfection (Fig. 1G). CD8+ T cells in the brain also expressed persistently reduced levels of the memory cell marker, IL-7Rα, whereas the cells in the spleen differentiated progressively into memory (IL-7Rα^hi^) cells (Fig. 1H). IL-2Rα expression was quickly lost on the OVA-specific CD8+ T cells in both brain and spleen (Fig. 1I). These results indicated that CD8+ T cells that migrate into the brain following activation in the periphery display a cell surface expression profile typical of early effectors, not memory cells, even at later time periods postinfection.

Because the experiments described above involved adoptive transfer of small numbers of OT-I cells, we addressed whether the results would be similar for endogenous OVA-specific CD8+ T cells. Following LM-OVA infection in the absence of OT-I cells, endogenous OVA-responsive CD8+ T cells migrated to various organs. OVA257–264-tetramer+ CD8+ T cells were found in the liver, lungs, brain, and spleen at 30 d postinfection (Fig. 2). Again, the OVA-specific CD8+ T cells in the brain were unique in exhibiting the lowest expression of CD62L and IL-7Rα. OVA-specific CD8+ T cells displayed similar IFN-γ expression in the spleen, lungs, and brain (Fig. 3B, 3C), despite the differences in CD62L and IL-7Rα expression. Proliferation of OVA-specific CD8+ T cells in the spleen and brain was also similar as measured by BrdU uptake (Fig. 3A), with cells displaying maximal proliferation around day 7 and return to homeostatic levels beyond day 15 postinfection. Thus, the cell surface profile of CD8+ T cells that homed to the brain is distinctly unique in comparison with CD8+ T cells in other lymphoid or nonlymphoid organs.

FIGURE 1. Ag-specific CD8+ T cells in the brain display a persistently activated phenotype in response to a peripheral infection. C57BL/6J mice were infected i.v. with LM-OVA (1×10^5). At various time intervals, spleens and brains were removed from mice after cardiac perfusion. A, Cell suspensions were prepared, and serial dilutions were plated on BHI agar plates to determine the bacterial burden (CFU/organ). B–I, C57BL/6J mice were injected i.v. with 1×10^5 OT-I TCR transgenic CD8+ T cells and challenged within 5–7 d with LM-OVA (1×10^5, i.v.). At various time intervals postinfection, spleens and brains were removed from mice after cardiac perfusion. B, Cells were stained with OVA-tetramer and anti-CD8 Abs and analyzed by flow cytometry. C, The numbers of OVA-tetramer+ CD8+ T cells in the brain and spleen were evaluated. D, To compare the rates of attrition, the percentage of cells remaining past day 5 postinfection were calculated, with numbers at the day 7 time point set as 100%. Cells were also stained with Abs against CD62L (E), CD11a (F), CD69 (G), IL-7Rα (H), and IL-2Rα (I), and the expression of these molecules on OVA-tetramer+ CD8+ T cells was evaluated in the brain and spleen. Each experiment involved the analysis of at least four mice/group and was repeated at least twice.

FIGURE 2. The endogenous CD8+ T cell response in various organs following LM-OVA infection. Spleens, lungs, livers, and brains were collected 30 d following i.v. infection of C57BL/6J mice with LM-OVA (1×10^5). No OT-I cells were used in this case. A, The numbers of OVA-specific CD8+ T cells in each organ were quantified by FACS analysis. B, The percentage of OVA-specific CD8+ T cells expressing high levels of CD62L and IL-7Rα was assessed by FACS analysis at various time points over a 35-d period following LM-OVA infection (1×10^5, i.v.). This was assessed from spleens, lungs, livers, brains, and peripheral blood (PBL). Each experiment involved analysis of at least four mice/group and was repeated at least twice.
and spleen (Fig. 4). Of the five mice that were rechallenged in the brain, four lost weight and died over the course of 6 d, and one gained weight and showed no sign of illness by the end of 10 d (Fig. 5A). We then immunized two groups of five mice with LM-OVA, as described in Fig. 1 (with $5 \times 10^3$ OT-1 splenocytes adoptively transferred prior to infection). Two weeks later, one group was depleted of CD8+ T cells using the anti-CD8 depleting Ab, and the second group was given a PBS injection. The mice were then rechallenged with LM-OVA (1,000) i.v. and given a second injection of anti-CD8 depleting Ab or PBS. All of the PBS-injected animals controlled the infection, showing low bacterial burdens in the brain at 3 d post rechallenge (Fig. 5B), and all had gained weight and appeared healthy prior to being sacrificed. In contrast, four of the five CD8-depleted mice had a high bacterial burden in the brain and had lost approximately one fifth (20.6%) of their weight, on average. A fifth CD8-depleted mouse, in line with the prior survival experiment, had a low bacterial burden and had gained weight.

We also examined the survival of LM-OVA–immunized mice in long-term experiments to assess the duration of protection provided by the immunization. Mice with $1 \times 10^3$ OT-1 CD8+ T cells adoptively transferred i.v. were subsequently infected i.v. with LM-OVA (10^5) to generate a memory CD8+ T cell response. To evaluate protection in the brain, mice were given an i.c. rechallenge (at days 60, 90, and 180) of LM-OVA. All of the naive mice succumbed to infection within the first week (Fig. 5C). Mice that were rechallenged i.c. with LM-OVA at day 60 after the primary infection survived. When vaccinated mice were rechallenged on day 90, ~50% survived; none of the vaccinated mice survived when rechallenged at day 180. Protection in the liver, lungs, and spleen was not abrogated at late time intervals in response to an i.v. or intranasal LM-OVA challenge (data not shown).

**CD49d and VCAM-1 govern the initial homing of CD8+ T cells to the brain**

The α4 integrin (CD49d) adhesion protein is well known to mediate the entry of T cells into the CNS in animal models of multiple sclerosis (33–35). We evaluated the role of CD49d in mediating the homing of OVA-specific CD8+ T cells to the brain following i.v. LM-OVA infection. Interestingly, anti-CD49d treatment of mice during initial priming resulted in a drastic reduction in the homing of OVA-specific CD8+ T cells to the brain (Fig. 6A). Migration of CD8+ T cells was not influenced by the absence of P-selectin glycoprotein ligand 1, P-selectin, ICAM, LFA1, CCR2, CCR5, or CCR6 (S. MacLean and S. Sad, unpublished observations). We then determined whether anti-CD49d Ab treatment had any influence on the CD8+ T cells that had immigrated to the brain prior to Ab treatment. At a time point (day 60) when memory CD8+ T cells were already formed in the periphery and CD8+ T cells were detectable in the brain, we began anti-CD49d Ab treatment. As is evident in Fig. 6B, the numbers of pre-existing CD8+ T cells in the brain were not modulated by anti-CD49d Ab treatment. These results indicated that CD49d function is not required for the entry or retention of CD8+ T cells in the brain at 60 d postinfection.

We evaluated the expression of CD49d on OVA-specific CD8+ T cells as they differentiated and migrated to the brain. The expression of CD49d was highest at day 5 postinfection (Fig. 6C), and cells that had migrated to the brain expressed the highest levels of CD49d. In both spleen and brain, the numbers of...
CD8<sup>+</sup> T cells decreased progressively to undetectable levels. Taken together, these results suggested that the expression of CD49d is high in Ag-specific CD8<sup>+</sup> T cells early on, and this is important for their trafficking to the brain. CD49d interacts with VCAM-1 in the brain on both inflamed endothelial cells (33, 34) and on epithelial cells of the choroid plexus (CP) (35) in the animal model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE). Therefore, we assessed whether blocking with an anti–VCAM-1 Ab following LM-OVA infection during the priming phase would have an effect similar to CD49d blocking. Indeed, this resulted in the blocking of OVA-specific CD8<sup>+</sup> T cell entry into the brain, comparable to the CD49d blocking (Fig. 6D). Stopping the blockade of CD49d–VCAM-1 after the first 25 d of treatment resulted in a rebound in the numbers of OVA-specific CD8<sup>+</sup> T cells in the brain, indicating that CD8<sup>+</sup> T cells may continue to cycle into the brain for ≥1 mo (Supplemental Fig. 1A). After the initial migration of OVA-CD8<sup>+</sup> T cells during the primary response, blockade of CD49d during LM-OVA rechallenge in the brain seemed to have little influence on the migration of CD8<sup>+</sup> T cells (Supplemental Fig. 1B, 1C).

**Localization of Ag-specific CD8<sup>+</sup> T cells in the brain**

We used an anti-CD8 Ab to deplete CD8<sup>+</sup> T cells in the systemic compartments. Because Abs do not cross the BBB, a depletion of brain-derived CD8<sup>+</sup> T cells would indicate that these cells may be in spaces accessible to blood. The main exception to Ab permeability in brain is in the CP, where IgG can freely pass through fenestrated blood vessels (36). Anti-CD8 Ab was injected at day 30 after LM-OVA infection, a time point when there is little systemic inflammation present, because *L. monocytogenes* is cleared rapidly by innate immune cells within the first week of infection (3). Higher doses of anti-CD8 Ab were required to delete cells in the brain compared with the spleen. When 1 μg of Ab was injected, there was a 1000-fold reduction in the numbers of OVA-specific CD8<sup>+</sup> T cells in the spleen, but no effect was noted on CD8<sup>+</sup> T cells in the brain (Fig. 4A, 4B). When the dose of anti-CD8 Ab was increased substantially, deletion of OVA-specific CD8<sup>+</sup> T cells in the brain was noted. At 2 h after Ab treatment, there was complete deletion of CD8<sup>+</sup> T cells in the spleen (∼99%), whereas only ∼30% of cells were deleted in the brain. At 24 h, complete depletion of OVA-specific CD8<sup>+</sup> T cells was observed in the brain (Fig. 4C).

We used frozen tissue sections to examine where in the brain OVA-specific T cells were located. In individual mouse brains examined at days 10 and 14 post-i.v. infection, CD45.1<sup>+</sup> OT-1 CD8<sup>+</sup> T cells that had been injected into CD45.1<sup>+</sup>CD45.2<sup>+</sup> hosts were observed primarily within the CP (Fig. 7A). These cells were also observed in the subarachnoid space (SAS) underlying the meninges surrounding the brain (Fig. 7B). Of the 53 cells that were positive for CD45.1 or CD8a observed in these two brains, 43 were in the CP, 10 were in the SAS, and none were in the parenchyma. In the CP, these T cells typically displayed a very polarized appearance (with the nucleus restricted to one side of the cell), in contrast to those found in the SAS, which exhibited CD45.1 staining more evenly distributed around the cell. Similar results were obtained when cells were stained with an anti-CD8a Ab (Supplemental Fig. 2). In the absence of an LM-OVA infection, this lymphocyte staining in the brain was not observed.
a mouse injected i.v. with naïve OT-1 CD8+ T cells (Supplemental Fig. 2).

We then determined where these CD8+ T cells (CD45.1) migrated to, in response to an i.c. rechallenge of LM-OVA. Previously vaccinated (104 LM-OVA, i.v.) mice were rechallenged on day 30 in the anterior forebrain with LM-OVA (103). Limited CD45.1 or CD8a staining was observed in the CP in individual brains at 3, 5, 7, and 10 d post-rechallenge. Rather, these cells were found in large clusters in CSF-filled spaces, including the ventricles (Fig. 7C) and SAS (Fig. 7D). Hundreds of CD8a and CD45.1+ cells were observed in these two locations in each brain. CD45.1+ cells were observed in the parenchyma around the injection site of LM-OVA (Fig. 7C, top inset), although not in large clusters. In mice that received a control saline brain injection instead of LM-OVA rechallenge, the CD45.1+ cells remained in the CP and were not observed to move out into regions where these cells had migrated to following LM-OVA rechallenge (Supplemental Fig. 3).

Activated OVA-specific CD8+ T cells can migrate from the parenchyma to the lymphoid compartment

We then went on to investigate the fate of OVA-specific T cells injected directly into the brain parenchyma. We wished to determine whether cells that were stimulated with LM-OVA would remain in the parenchyma or migrate out of the brain upon transfer. We injected recipient mice simultaneously with a mixture of cells that were stimulated in vitro with LM-OVA 3 d (CD45.2+) or 12 d (CD45.1+) prior to transfer. In both cases, CD8+ T cells were purified, mixed 1:1, and injected (~1 × 106 cells) into CD45.2+ C57BL/6J hosts (Fig. 8A). Although a million cells were transferred, far fewer cells were recovered at 24 h after transfer, with the recently stimulated cells displaying better initial take. Beyond day 1, cells underwent expansion followed by a protracted and total contraction in the brain (Fig. 8B). At day 1 after transfer, cells were not detectable in the spleen. Interestingly, recently stimulated cells were detectable at day 7 in the spleens, indicating that activated CD8+ T cells have the potential to migrate out of the parenchyma back into the peripheral circulation. Cells that migrated to the spleen underwent little contraction subsequently, whereas their counterparts that remained in the brain underwent protracted and massive contraction (Fig. 8B). More interestingly, in the day-3 cells, although the CD8+ T cell population in the brain remained CD69hi, the injected OT-1 cells found in the spleen were CD69low (Fig. 8C). These results indicated that the same population undergoes differential contraction in the spleen and brain, which correlates with the phenotype of the cells in each organ. Cells that migrated from the brain to the spleen persisted and expressed undetectable levels of CD69, whereas cells that remained in the brain underwent contraction and expressed high levels of CD69.

To find where the OT-1 cells injected into the parenchyma can migrate to, we injected in vitro-activated OT-1 CD8+ T cells, as described in Fig. 8 (3 d postactivation), to track the fate of these cells by immunohistochemistry. Approximately one million cells were injected into the anterior forebrain of naïve mice (Fig. 9A). At 1–3 d postinjection, clusters of cells were observed in only two
repeated at least twice. Each experiment involved the analysis of at least four mice/group and was evaluated in the spleen and brain of recipient mice by flow cytometry.

These cells had a polarized morphology similar to those observed in the CP in response to an i.v. LM-OVA infection (Fig. 7). Cells were located both inside and outside of the CP (Fig. 9D). They were not observed on the side contralateral to the injection. Within the lateral ventricle where they were present, cells were mainly found in CSF-filled spaces. The CSF-filled spaces included the SAS locations: the region adjacent to the bottom of the injection tract (Fig. 8, B–D) and 25 μm (C, D). Two mice each were evaluated at 1 and 3 d postinjection, and staining was repeated at least twice.

FIGURE 9. Ag-specific CD8⁺ T cells injected into the parenchyma can migrate into the CP. OVA-specific CD8⁺ T cells were generated, as described in Fig. 8, and used at day 3 following activation. A. Horizontal profile of an embedded, sectioned mouse brain injected with activated OT-1 CD8⁺ T cells. The injections were placed in the upper left region of the brain (red circle). B. A cluster of CD45.1⁺ cells (shown) and CD8⁺ cells (not shown) were easily observable near the bottom of the injection tracts. Outside of the injection tracts, cells were mainly found in CSF-filled spaces. In the lateral ventricle on the side ipsilateral to the injection, CD45.1⁺ (shown) and CD8⁺ (not shown) cells could be found entering into and within the CP at 1 d (C) and 3 d (D) postinjection. Scale bars, 50 μm (B) and 25 μm (C, D). Two mice each were evaluated at 1 and 3 d postinjection, and staining was repeated at least twice. lv, lateral ventricle; 3rd v, third ventricle.

nonlymphoid areas, and memory T cells in these areas (effector-memory cells) may provide rapid protection against the pathogen. However, nonlymphoid organs vary in physiology and function (37, 38). Therefore, it is pertinent to ask whether similar T cell-trafficking and -homing mechanisms apply to all nonlymphoid organs. The brain is a nonlymphoid organ that is uniquely separated from systemic traffic by the BBB and the blood–CSF barrier (BCSFB). Thus, T cells that immigrate to the brain will have to cross an extra hurdle. Therefore, important questions are how and under what circumstances do T cells home to the brain and how long do T cells persist there to mediate protection.

The selective migration of a subset of activated T cells (effector-memory) to nonlymphoid organs has been shown in various models (20, 21). These T cells express reduced levels of the lymph node-homing receptor CD62L and chemokine receptor CCR7 (21) and mediate effector functions rapidly (24). Our results indicated graded expression of CD62L in differentiating CD8⁺ T cells (Fig. 2), and this most likely influences the subsequent migration of the cells (37). An important aspect of CD8⁺ T cells in the brain was their persistently elevated expression of CD69, a marker of early activation. Indeed, T cells in the brain have been shown to express high levels of CD69 following viral infections (39, 40). CD69⁺ CD8⁺ T cells are generated immediately after activation by LM-OVA, and such cells are undetectable in the spleen and other lymphoid organs by day 7 postactivation (41). Our results suggested the possibility that rather than being deleted rapidly, CD69⁺ CD8⁺ T cells continue to circulate through the brain.

Our results indicated that the initial homing of CD8⁺ T cells to the brain is primarily dependent on CD49d and VCAM-1. In the brain, VCAM-1 is expressed in the CP epithelium and parenchymal blood vessels (35, 42). Because blocking CD49d function had no effect on the brain CD8⁺ T cell population by 60 d following infection, the cells either eventually became noncirculating in the peripheral lymphoid organs by day 7 postactivation (41). Our results suggested the possibility that rather than being deleted rapidly, CD69⁺ CD8⁺ T cells continue to circulate through the brain.

FIGURE 8. Activated CD8⁺ T cells that migrate out of the brain undergo a reduced contraction in the periphery. OT-1 spleen cells were stimulated in vitro for 3 d (CD45.2⁺) or 12 d (CD45.1⁺), as described in Materials and Methods. A, CD8⁺ T cells were quantified and mixed 1:1 and injected i.c. (∼10⁶/mouse) into the anterior forebrain of naive C57BL/6J recipients. At various time intervals, the numbers of CD45.1⁺ and CD45.2⁺ OVA-specific CD8⁺ T cells (B) and the expression of CD69 (C) were evaluated in the spleen and brain of recipient mice by flow cytometry. Each experiment involved the analysis of at least four mice/group and was repeated at least twice.

Discussion

Subsequent to the initial pathogen encounter, <10% of primed CD8⁺ T cells persist as memory cells (11, 14). Subsets of these memory T cells home to lymphoid (central memory cells) and nonlymphoid (effector memory cells) organs to mediate protection against a subsequent encounter with the same pathogen (21, 27). From a host’s perspective, this model of T cell differentiation makes sense because pathogen encounters may occur often in the brain and CSF-filled spaces. The CSF-filled spaces included the SAS locations: the region adjacent to the bottom of the injection tract (Fig. 9B–D). They were not observed on the side contralateral to the injection. Within the lateral ventricle where they were present, cells were mainly observed outside of the CP (Fig. 9C, 9D). These cells had a polarized morphology similar to those observed in the CP in response to an i.v. LM-OVA infection (Fig. 7). Cells remaining inside the CP were still easily observable by 3 d postinjection (Fig. 9D).

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residents in the brain or entered in low numbers through a CD49d-independent mechanism at this later time. Our preliminary evidence indicated that CD8+ T cells continue to cycle into the brain for ≥1 month following systemic infection (Supplemental Fig. 1A). However, we have not been able to determine whether some of this entry is CD49d/VCAM-1 independent. Further work with selective VCAM-1 ablation in a transgenic model would likely be necessary to determine this precisely.

The requirement for CD49d as a key mediator of homing of Ag-specific CD8+ T cells to the brain is consistent with its role in EAE (33). CD49d forms the α-chain of α4β1 integrin (VLA-4), which is expressed on most peripheral lymphocytes, thymocytes, and monocytes (43). α4β1 integrins interact with VCAM-1 to mediate cell–cell interactions (43–45). Abs against α4β1 integrins were shown to prevent EAE in mice (33), and an inhibitory α4 integrin Ab is one of the most potent drugs used to stem the progression of multiple sclerosis in humans (31). It would be of interest to determine whether the role of α4 integrin mediating T cell entry into the brain changes over time in multiple sclerosis, because this may have implications for drug efficacy. It is interesting that the initial immune surveillance in the brain following a peripheral infection. Following a reinfection in the brain of immunized mice, the Ag-specific CD8+ T cells were found to have expanded in the ventricles and SAS and entered into the brain for ~20 d. As the investigators of that study pointed out, and in agreement with our study, long-term resident CD103+ cells did not form in the absence of Ag in the brain. Indeed, we only found CD103+ CD8+ T cells in the brain following a rechallenge of LM-OVA injected into the brain (Supplemental Fig. 4). CD103 is not expressed on brain CD8+ T cells in the case of a systemic LM-OVA infection in which Ag is not present in the brain (47) (K. Young, S. MacLean, S. Sad, unpublished observations).

Our study indicated that although activated T cells home to CP, they are progressively eliminated over the period of a few months in the mouse brain. Because these cells do not express memory markers, it is understandable that they do not persist. We propose that effector T cells, not memory cells, play a key role in mediating immune surveillance in the brain following a peripheral infection. This subset of T cells likely circulates through the BCSFB, a normal route of T cell entry into the noninflamed CNS. Only following infection within the brain do these cells undergo a secondary activation and migrate into the parenchyma. The lack of expression of memory markers on CD8+ T cells in the brain may ensure that the cells that traffic to the brain do not persist there for the host’s lifetime, thereby avoiding undesirable inflammation of the brain every time a host encounters an infection in the peripheral compartment.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplemental Figure 1. Prolonged CD49d blocking and CD49d blocking during a brain re-challenge does not prevent antigen-specific CD8$^+$ T cell entry. (A) Approximately 500 CD45.1$^+$ OT-1 CD8$^+$ T cells were adoptively transferred into CD45.2$^+$ host mice, which were subsequently infected with $10^4$ LM-OVA i.v. Starting at two days following the infection, four mice were given i.p. injections of CD49d and VCAM-1 blocking antibodies (100 μg total antibody/injection), and four mice were given PBS for a control group. The injections of blocking antibodies were rotated between a 1:1 mix of the two anti-CD49d blocking antibodies for two days, then anti-VCAM-1 blocking antibody on the third day, with this rotation being performed up to day 25 post-infection. No injections were given on days 10, 17 and 24. Two mice from the antibody injected group and two from the control group were sacrificed on day 15 to analyze the numbers of OVA-specific CD8$^+$ T cells in their brains at this time-point. After day 25, the remaining mice were left with no further treatments until being sacrificed for FACS analysis at day 34 post-infection. At day 15, mice that had received the blocking antibodies had a barely detectable population of OVA-specific CD8$^+$ T cells in their brains. The number of cells in the brains following the halting of blocking antibody injections, at day 34, was similar to the number found in the brains of control animals that had been injected with PBS. We interpret this as indicating that virtually all of the OVA-specific CD8$^+$ T cells in the brain at day 34 post-infection had entered during a period after which the blocking antibodies would have lost their efficacy, beyond day 25 post-infection. Alternatively, most or all cells CD8$^+$ T cells entering at this time-point represent a population of late-entering cells that enter via a mechanism independent of CD49d and VCAM-1. Either explanation necessitates that a turnover of OVA-specific
CD8+ T cells has occurred in the brain. (B) Mice which had 1 x 10^4 CD45.1+ OT-1 splenocytes adoptively transferred i.v. were infected i.v. with 1 x 10^4 LM-OVA, and re-challenged with 1 x 10^3 LM-OVA i.c. 36 days later. One group of mice was injected with CD49d blocking antibodies on the day prior to the i.c. re-challenge, the day of the re-challenge, and the three subsequent days. All of the mice were sacrificed four days following the i.c. re-challenge in order to determine the numbers of OVA-specific CD8+ T cells in their brains. (A) All of the mice had several thousand OVA-specific CD8+ T cells in the brain at four days following the re-challenge, with those that had been injected with the CD49d blocking antibodies displaying a similar number of these cells. (B) In animals that were not re-challenged at this time point, the numbers of OVA-specific CD8+ T cells were over 10-fold lower. Again, no difference was observed between the numbers of these cells in mice treated with anti-CD49d blocking antibodies, or control animals.
Supplemental Figure 2. **CD45.1$$^+$$ cells in the choroid plexus at various time points following an intravenous LM-OVA infection.** (A) CD45.1 staining detected many highly polarized cells within the stroma of the choroid plexus at day 10 following an LM-OVA infection of CD45.1$$^-$$, CD45.2$$^+$$ mice that had previously been injected with CD45.1$$^+$$ OT-1 lymphocytes. Similar staining was observed using a CD8a antibody (B). Detection of these cells was similar at day 14 following the LM-OVA injection (C). At day 60 following the LM-OVA injection, only a few CD45.1$$^+$$ cells (or CD8$$^+$$ cells; not shown) could be found, and these cells had a much less polarized appearance. (E) In a mouse that had been injected with CD45.1$$^+$$ OT-1 lymphocytes, then sacrificed 14 days later without receiving a bacterial injection, no discreet CD45.1 staining was observed in the choroid plexus. Scale bars = 20 μm in all panels. Individual mice were evaluated at each time point, and staining was repeated at least twice.
Supplemental Figure 3. CD45.1⁺ lymphocytes in the medial longitudinal fissure of a vaccinated mouse re-challenged in the brain, and the choroid plexus of a non-challenged vaccinated mouse. C57BL/6J mice were injected with OT-1 cells and challenged i.v. with LM-OVA (10⁵). At day 60, mice received LM-OVA (10⁵) or saline given intracranially. In a CD45.1⁻, CD45.2⁺ mouse previously injected with CD45.1⁺ OT-1 lymphocytes, the CD45.1⁺ lymphocytes were found mainly in the CSF-filled regions of the brain by seven days post-intracranial infection with LM-OVA. Shown is a picture of a cluster of CD45.1⁺ and CD45.1⁻ cells massed in the medial longitudinal fissure (A), where large numbers of infiltrating cells were found at this time point. In a control brain that received a saline intracranial injection, no CD45.1⁺ cells were found to have infiltrated into the CSF or parenchyma, and no infiltrating cells were found in the medial longitudinal fissure (B). CD45.1 staining was observed in the choroid plexus from this mouse (C). Scale bars = 25 μm, and applies to all panels. Individual mice were evaluated for each treatment, and staining was repeated at least twice.
Supplemental Figure 4. CD103 expression on lymphocytes in the brain following an LM-OVA re-challenge in the brain. C57BL/6J mice with adoptively transferred CD45.1+ OT-1 splenocytes (1 x 10^4) were infected i.v. with 1 x 10^4 LM-OVA, then re-challenged with intra-cranial injections of 1 x 10^3 LM-OVA 30 days later. Shown is CD103 staining in brains collected from mice 10 days (A, B) and 7 days (C) post-re-challenge. (A) A lymphocyte in the brain parenchyma near the re-challenge injection site, showing CD103 and CD45.1 staining (with individual staining in A’ and A’’). (B) Another CD45.1+ cell in the same region that was CD103-. (C) Lymphocytes in a brain fissure, showing staining for CD103 and CD8 (with individual staining in C’ and C’’). Scale bars = 25 μm.