Immunopathology and Infectious Diseases

Rapid Formation of Extended Processes and Engagement of Theiler's Virus-Infected Neurons by CNS-Infiltrating CD8 T Cells

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A fundamental question in neuroimmunology is the extent to which CD8 T cells actively engage virus-infected neurons. In the Theiler's murine encephalomyelitis virus (TMEV) model of multiple sclerosis, an effective central nervous system (CNS)-infiltrating antiviral CD8 T cell response offers protection from this demyelinating disease. However, the specific CNS cell types engaged by these protective CD8 T cells in TMEV-resistant strains remains unknown. We used confocal microscopy to visualize the morphology, migration, and specific cellular interactions between adoptively transferred CD8 T cells and specific CNS cell types. Adoptively transferred GFP+ CD8+ splenocytes migrated to the brain and became 93% specific for the immunodominant virus epitope D^b:VP2₁₂₁₋₁₃₀. These CD8 T cells also polarized T cell receptor, CD8 protein, and granzyme B toward target neurons. Furthermore, we observed CD8 T cells forming cytoplasmic processes up to 45 μ m in length. Using live tissue imaging, we determined that these T cell-extended processes (TCEPs) could be rapidly formed and were associated with migratory behavior through CNS tissues. These studies provide evidence that antiviral CD8 T cells have the capacity to engage virus-infected neurons *in vivo* and are the first to document and measure the rapid formation of TCEPs on these brain-infiltrating lymphocytes using live tissue imaging. (*Am J Pathol* 2010, 177:1823–1833; DOI: 10.2353/ajpath.2010.100231)

tions and potentiate immune-mediated neuropathology. However, the capacity of neurons to accommodate the formation of traditional immune synapses required for engagement by antiviral CD8 T cells remains controversial due to their reduced capacity to translate MHC class I molecules. It has been demonstrated that neurons express little or no detectable levels of Major histocompatibility complex (MHC) class I protein despite high levels of mRNA expression.^{1–5} Other studies have concluded that MHC class I molecules can be up-regulated in neuronal cultures.^{6,7} An additional role for class I molecule expression in neuronal development has also been put forward in that genetic deletion of MHC class I genes results in significant deficits in synaptic plasticity.⁸ The conclusion from the above studies implies that underlying class I expression is necessary for full physiologically functioning neurons. However, translation of class I protein by neurons *in vivo* may be below detection through conventional means.

To explore the possibility of traditional immune synapse formation between CD8 T cells and neurons, we examined this interaction in the Theiler's murine encephalomyelitis virus (TMEV) model of multiple sclerosis. Resistance to TMEV-induced demyelinating syndrome is dependent on the generation of a potent antiviral CD8 T cell response and the expression of specific MHC class I molecules.^{9,10} Mice with H-2 haplotype b, d, and k clear TMEV infection and do not develop chronic demyelinating disease. Meanwhile, mice of H-2 haplotype f, p, q, r, s, and v are susceptible to chronic TMEV infection and progressive demyelination.^{9,10} In addition to the genetic

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The direct engagement of neurons by CD8 T cells is a putative mechanism to both clear neurotropic virus infec-

linkage to MHC class I molecules, resistance to TMEV-induced demyelination is conferred through expansion of CD8 T cells specific for the immunodominant virus peptide, VP2₁₂₁₋₁₃₀, presented in the context of the D^b class I molecule.¹¹⁻¹³ Despite the importance of this interaction in protecting against demyelination, the specific central nervous system (CNS) cell type(s) engaged by antiviral CD8 T cells remains undefined. Among the CNS cell types potentially engaged by CD8 T cells, neurons would pose a considerable challenge for T cell receptor engagement due to their reduced expression of class I molecules as previously reported.^{1,14,15} In this study, we use an adoptive transfer technique that enables, with high confidence, the imaging of antiviral CD8 T cell responses in the CNS. In this analysis, we determined the extent antiviral D^b:VP2₁₂₁₋₁₃₀ epitope specific CD8 T cells engage specific TMEV infected CNS cell types. We also use this highly efficient transfer strategy to visualize CD8 T cell morphology and motility in the CNS tissue using live tissue imaging.

Materials and Methods

Mice

C57BL/6-Tg(UBC-GFP)30Scha/J (stock number 004353) females were bred in-house in the University of Cincinnati LAMS facility. Five-week-old C57BL/6J female mice were obtained from Jackson labs (stock number 000664). All animals were used according to University of Cincinnati and Children's Hospital Medical Center LAMS- and IACUC-approved protocols.

Adoptive Transfer

Spleens of GFP+ mice were removed and strained through a nylon mesh 100- μ m filter. CD8+ cells were purified from the resulting lymphocyte population using MACS LS cell purification columns (Miltenyi Biotec, Auburn, CA) according to the manufacturer's protocol, resulting in 95% purity as determined by flow cytometric analysis (data not shown). C57BL/6 mice were irradiated with 400 rads of γ -radiation then received 10⁶ CD8+ positively sorted spleen cells via tail-vein injection. A pilot experiment determined that irradiation enabled roughly fivefold higher levels of GFP+ cells to be transferred (data not shown). This transfer was performed in four mice over two separate experiments.

Virus Infection

One day after adoptive transfer, mice were anesthetized with isoflurane and intracranially infected with the 2 $\times$ 10⁶ PFU of Daniel's strain TMEV.¹⁶ Animals were euthanized 7 days after TMEV infection and tissue was collected.

Tissue

The brains of 7-day TMEV-infected animals were removed and divided into hemispheres. The left hemisphere was analyzed using flow cytometry, and the right hemisphere was used for immunohistochemistry.

Reagents

D^b:VP2₁₂₁₋₁₃₀ and D^b:E7 tetramers were made as previously described.^{15,17,18} H-2D^b and human β 2m expression vectors were generous gifts from John Altman and Mark Davis, respectively. Protein products expressed in bacteria from these plasmids were isolated and folded in excess VP2₁₂₁₋₁₃₀ or E7 peptide to create monomer complexes. Monomer complexes were then desalted with PD-10 desalting columns (Pharmacia, Upsala, Sweden) then biotinylated using a BirA biotinylation kit (Avidity, Denver, CO), purified with Mono Q cation exchange columns (Biorad, Hercules, CA), and complexed with APC-streptavidin (Molecular Probes, Eugene, OR) at a 4.1:1 molar ratio. Tetramers were then purified by S-200 size exclusion gel filtration (Biorad, Hercules, CA). Rabbit anti-TMEV antibody was generously provided by Moses Rodriguez (Professor of Neurology and Immunology, Mayo Clinic, Rochester, Minnesota). Rabbit anti-mouse granzyme B antibody was generously provided by Markus Simon (Professor of Immunology, Max-Planck Institute of Immunobiology, Freiburg, Germany). All other antibodies used in this study are listed in Table 1.

Isolation of Brain Lymphocytes

Brains were strained through a nylon mesh 100- μ m filter into RPMI (MediaTech Inc., Herndon, VA), and 700 μ g of collagenase type 4 (Worthington, Lakewood, NJ) was added to each 5-ml tissue suspension. Suspensions were then incubated in a water bath at 42°C for 45 minutes. Each 5-ml suspension was then added to 50 ml high speed centrifuge tubes (Nalge Nunc International, Rochester, NY) containing a solution of 1 ml of 10 $\times$ PBS, 9 ml of Percoll (Sigma-Aldrich GmbH, Steinheim, Germany), and 35 ml of RPMI. Cell suspensions were then spun at 10,000 rpm (Sorvall SS-34 rotor) for 30 minutes ($\times g$ max = 11962.6). A lymphocyte layer present in the bottom 5 ml of solution was collected. The lymphocyte layer was then resuspended into 50 ml conical tubes (Becton Dickinson, Franklin Lakes, NJ) and diluted with RPMI to a total volume of 50 ml. Cell sus-

Table 1. Antibodies Used for Immunohistochemistry and Confocal Microscopy

Antibody	Manufacturer	Catalog number
Chicken anti- β III Tubulin	Chemicon	AB9354
Hamster anti-mouse TCR β	Biolegend	109202
Mouse anti-NeuN	Chemicon	MAB377
Hamster anti-mouse CD3	AbD Serotec	MCA2690
Rat Anti-mouse CD8	BD Pharmingen	553031
Rat anti-mouse CD8	AbD Serotec	MC2694
Mouse anti-GFAP Cy3	Sigma Aldrich	C9205
Mouse anti-GFP	Chemicon	MAB3580
Mouse anti-human granzyme B	Caltag	GRB00
Rat anti-mouse CD18	GeneTex	GTX74602
Goat anti-mouse alexafluor 488	Invitrogen	A11029
Goat anti-rabbit alexafluor 546	Invitrogen	A11035
Goat anti-rat alexafluor 555	Invitrogen	A21434
Goat anti-hamster alexafluor 647	Invitrogen	A21451
Goat anti-rabbit alexafluor 488	Invitrogen	A31627
Goat anti-rabbit alexafluor 647	Invitrogen	A21443
Goat anti-chicken Alexafluor 647	Invitrogen	A21449

pensions were then spun at $300 \times g$ for 10 minutes on a Sorvall Legend RT tabletop centrifuge. Media was aspirated off, and cell pellets were resuspended in RPMI media.

FACS

Isolated brain lymphocytes were resuspended in FACS buffer (1X PBS, 1% FCS and 0.025% sodium azide) and incubated with D^b:VP2₁₂₁₋₁₃₀ tetramer or D^b:E7 tetramer for 40 minutes followed by a 20 minute incubation with anti-CD4 and anti-CD8. Cells were then rinsed twice with FACS buffer and fixed in 1% PFA in 1X PBS. Samples were run on a BD LSR II flow cytometer (BD Biosciences, San Jose CA) and analyzed using FACS Diva software (BD Biosciences, San Jose CA).

Immunohistochemistry

Fixed and fresh frozen brain tissue slices were used for these studies. For fixed tissue slices, brain hemispheres were incubated in 2.5% PFA and 4% sucrose overnight. Tissue was then incubated in 10% sucrose for 8 hours, 20% sucrose for 24 hours, and 30% sucrose for 48 hours. Tissue was then frozen in -20°C isopentane. Sixty-micron thick tissue slices were cut on a cryostat and placed onto positively charged slides (VWR International, Batavia, IL). Fresh frozen coronal slices containing the hippocampal region were cut and adhered to slides. Slides were incubated with primary antibodies overnight followed by an overnight rinse. Slides were then incubated with secondary antibodies for 4 hours followed by an overnight rinse in PBS. Because TCR β and MHC class I antibodies had low efficacy in fixed tissue, fresh frozen tissue was used. For fresh frozen tissue slices, brain hemispheres were snap frozen on dry ice. Fourteen-micron-thick tissue slices were cut on a cryostat and placed onto charged slides (VWR International, Batavia, IL). Tissue was then fixed in -20° 95% ethanol for 15 minutes. Slides were incubated with primary antibodies overnight followed by 4×5 minute rinses. Slides were then incubated with secondary antibodies for 1 hour followed by 4×5 minute rinses.

Confocal Analysis

Images for figures and cellular reconstructions were collected using a Leica (Wetzlar, Germany) SP5 confocal microscope set up on a DMI 6000 inverted microscope equipped with a 63x oil immersion objective (NA, 1.4). LAS AF version 2.0.0 software (Leica Microsystems CMS GmbH, Mannheim, Germany) was used to analyze images. Three-dimensional Z-series stacks were captured at $0.25 \mu\text{m}$.

Time-Lapse Confocal Imaging

Briefly, hippocampal brain slices were prepared for time-lapse confocal imaging by anesthetizing animals with

ketamine (100 mg/kg) followed by decapitation. Brains were removed, immersed in ice-cold artificial cerebral spinal fluid (ACSF) (124 mmol/L NaCl, 3 mmol/L KCl, 2 mmol/L MgSO₄, 2 mmol/L CaCl₂, 1.5 mmol/L NaH₂PO₄, 26 mmol/L NaHCO₃, 10 mmol/L glucose, osmolarity adjusted to ~ 300 mOs, pH 7.3–7.4) for 2–3 minutes, and glued to a metal tissue chuck. Transverse slices $400 \mu\text{m}$ thick were cut on a tissue slicer (Campden Instruments) in ice-cold ACSF. Slices were then transferred into a small beaker containing ACSF bubbled continuously with 95% O₂ and 5% CO₂. The slices were then allowed to equilibrate for approximately 60 minutes at room temperature. Slices were transferred into a glass bottom microscope stage chamber continuously perfused with oxygenated ACSF (95% O₂, 5% CO₂) at 36°C . The microscope was also encased in a luidin chamber warmed to 36°C to maintain temperature stability. Slices were screened under epifluorescent illumination and $\times 10$ (NA 0.3) magnification to locate GFP-expressing lymphocytes. Magnification was then increased to $\times 40$ (NA 0.6) or $\times 63$ (NA 1.4) to focus on specific cell(s) of interest and the experiment commenced.

Results

Adoptively Transferred Brain Infiltrating CD8 T Cells Are Highly Specific for TMEV

CD8 T cell specificity for the immunodominant D^b:VP2₁₂₁₋₁₃₀ TMEV epitope in the C57BL/6 mouse strain during acute TMEV infection has been well characterized.^{11–13,15,19,20} To determine the target of these antiviral CD8 T cells in the CNS during acute TMEV infection, we adoptively transferred CD8+ GFP+ splenocytes into partially irradiated mice according to our protocol outlined in Figure 1A. Mice were TMEV-infected one day later and euthanized at 1 week. For each mouse, the left hemisphere was analyzed by flow cytometry for efficacy of adoptive transfer. The right hemisphere was analyzed by confocal microscopy. Through this analysis, we determined that adoptively transferred GFP+ CD8+ cells became highly specific for the immunodominant D^b:VP2₁₂₁₋₁₃₀ epitope. Ninety-two percentage of GFP expressing cells recovered from the CNS also expressed CD8 protein (Figure 1B). Of this GFP+ CD8+ population, approximately 93% of CD8+ cells were specific for the D^b:VP2₁₂₁₋₁₃₀ epitope (Figure 1B). This high level of epitope dominance was not the result of nonspecific binding to tetramer, as GFP+ CD8+ cells did not appreciably stain with mock control D^b:E7 tetramer (Figure 1C) consistent with previous reports.^{17,21} This high percentage of adoptively transferred CD8+ cells that became specific for the D^b:VP2₁₂₁₋₁₃₀ epitope provided us with high confidence that GFP expression served as a suitable marker for detection of virus-specific CD8 T cells in the CNS using confocal microscopic analysis of the adjacent hemisphere.

Figure 1. Adoptively transferred GFP+ CD8+ splenocytes infiltrate the CNS and become highly focused toward the immunodominant D β :VP2121-130 virus epitope during acute TMEV infection. **A:** Schematic for adoptive transfer system to analyze CNS-infiltrating CD8 T cells using immunohistochemistry and flow cytometry. Magnetically purified spleen-derived CD8+ cells (1×10^6) isolated from GFP-expressing C57BL/6 mice were intravenously administered to partially irradiated wild-type C57BL/6 mice. One day later, mice were intracranially infected with TMEV. At 7 days postinfection, brain tissue was harvested. The left hemisphere was analyzed by flow cytometry for efficacy of adoptive transfer. The right hemisphere was prepared for immunohistological staining. **Arrows** denote site of irradiation, adoptive transfer, intracranial infection, and brain harvest. Adoptively transferred GFP+ cells were analyzed for CD8 expression and D β :VP2121-130 tetramer (**B**) or negative control D β :E7 tetramer (**C**) staining. FACS Results are representative of four animals from two separate experiments.

Translation of TMEV Protein Is Predominately in Neurons During Acute Infection

We determined through flow cytometric analysis of the left hemisphere that 93% of adoptively transferred GFP+ CD8 T cells were antiviral (Figure 1B). We next sought to determine the major cell type(s) that translate(s) virus protein and therefore served as a potential target of antiviral CD8 T cells through analysis of tissue slices obtained from the opposing right hemisphere. Previous analysis of chronic infection of the SJL mouse strain had concluded using peroxidase methods of immunohistochemistry that neurons and astrocytes in the spinal cord were positive for TMEV infection.²² Using confocal microscopy, we observed TMEV protein translation in the hippocampus, striatum, hypothalamus, and cortex of acute TMEV infected C57BL/6 mice. At random, we counted 150 TMEV-positive cell bodies from 10 tissue slices obtained from the hippocampus of four mice receiving adoptive transfer of CD8+GFP+ cells (Figure 1). One hundred forty-two of these cell bodies also stained

positive for the neuronal marker NeuN (94.7%). This demonstrated that under acute TMEV infection, neurons were the primary cell type translating viral protein (Figure 2, A–C). In addition, 150 GFAP-positive cells from 10 tissue slices through the hippocampus from four animals were randomly analyzed for TMEV staining. Among these cells, none stained positive for TMEV protein, indicating activated astrocytes were not actively translating virus protein (Figure 2, D–F). Furthermore, TMEV infected neurons often presented with dendritic beading indicative of cellular degeneration (Figure 2, G–I). This analysis demonstrated that acute TMEV infection in C57BL/6 mice is predominately localized to neurons.

CD8 T Cells Form Immune Synapses with TMEV Infected Neurons

Adoptively transferred CNS infiltrating GFP+ CD8 T cells were observed in close proximity to TMEV infected neurons and their processes (Figure 3A). This observation prompted an analysis of the molecular interaction between CD8 T cells and TMEV-infected neurons. Interestingly, CD8 T cells did not readily polarize CD3 molecule toward virus-infected neurons when in close proximity (Figure 3, B–E). Instead, CD8 T cells polarized both CD8 protein and T cell receptor toward neurons in brain regions with known TMEV infection, an observation previously defined to be indicative of immune synapse formation (Figure 3, F–M).²³ CD8 T cells in close proximity to TMEV infected neurons also polarized granzyme B (Figure 3, N–Q). Despite T cell receptor and granzyme B polarization among proximal CD8 T cells, MHC class I H-2D β protein was not highly expressed on neurons (Figure 4). By comparison, class I staining was readily detectable on directly adjacent CD8+ cells (Figure 4, A–H). Analysis of the fluorescent intensity of CD8 protein through tracing of the cell membrane revealed measurably higher concentrations near points of engagement of CD8 T cells with targeted neurons (Figure 5, A–C).

Adoptively Transferred CD8 T Cells Are Morphologically Diverse and Activated

Readily apparent among GFP+ CNS infiltrating CD8 T cells were numerous distinct morphologies. Morphologies included round GFP+ cells (Figure 6, A–D and I–L) and GFP+ cells that formed long projections of varying thickness, which we have termed T cell-extended processes (TCEPs) (Figure 6, E–H and M–P). In Figure 6E, the GFP+ cell shown presents with a TCEP that extends to nearly 45 μ m in length. Both round and TCEP-presenting GFP+ cells expressed CD3 (Figure 6, B and F) and CD8 (Figure 6, J and N) protein, confirming their identity as T lymphocytes as predicted by the flow cytometric analysis on the opposite brain hemisphere (Figure 1B). Additionally, both round and TCEP-presenting GFP+ cells presented with an activated phenotype as indicated by granzyme B expression (Figure 6, Q–X).

Figure 2. TMEV primarily infects neurons during acute infection in C57BL/6 mice. Neurons are the primary CNS cell type expressing virus protein during acute TMEV infection. Z-stack 3D reconstructions revealed that TMEV-infected cells were predominantly neurons as determined by positive staining with the neuronal marker NeuN. Representative labeling for TMEV (A) and NeuN (B) demonstrated a high level of overlap in merged images (C). Labeling for TMEV (D) never overlapped with GFAP labeling (E) as shown by merged images (F). Results are representative of an analysis of hippocampus in four TMEV-infected mice. Neurons that stain positive for TMEV protein presented with the onset of dendritic beading (G), while others were heavily degenerated (H) and fragmented (I) (results are representative of an analysis of hippocampal regions in 10 TMEV-infected mice). Scale bars = 25 μ m.

CD8 T Cell Phenotype Is Associated with Motility within the CNS

Additional immunohistological staining revealed that CD18, a component of the LFA-1 adhesion molecular complex, was often highly expressed at the tip of the TCEP on GFP+ cells indicative of cell adhesion (Figure 7, A and B). The expression pattern of CD18 and the observation of TCEPs being formed by various CD8 T cell morphologies prompted a comprehensive analysis of the brain using live tissue imaging. High endogenous GFP expression among adoptively transferred CD8+ cells enabled clear visualization using real-time confocal analysis in live hippocampal sections (Figure 1B). Through this analysis, the potential importance of the different CD8 T cell morphologies became apparent. Round GFP+ cells (previously defined in Figure 6, A–D and I–L above) were stationary and maintained a spherical shape for over one hour (Figure 7C; please see Supplemental Movie 1 at <http://ajp.amjpathol.org>). GFP+ cells that presented with large segmented TCEPs (previously defined in Figure 6, U–X above) were motile and traveled through tissue at a rate of approximately 1–2 μ m/min. The specific cell shown in Figure 7D used a “stop-and-go burst pattern” of movement using TCEP formation to accomplish motion (please see Supplemental Movie 2 at <http://ajp.amjpathol.org>). The final GFP+ cell phenotype observed for motility was consistent with the previously analyzed morphology in which the TCEP was extended from the defined cell

body (Figure 7, A and B; please see Supplemental Movie 3 at <http://ajp.amjpathol.org>). This cell began round and in the course of under 2 minutes produced a TCEP of approximately two full cell lengths which could be contracted and re-extended in the opposite direction (Figure 7E).

Discussion

In this study, we have developed an adoptive transfer technique enabling with high confidence the analysis of molecular interactions, regional location, morphology, and motility of a polyclonal virus epitope-specific CD8 T cell response in the CNS. We have also determined that TMEV resides primarily within neurons during acute infection of C57BL/6 mice. In this model, CD8 T cells were observed tightly apposed to target neurons. Tightly apposed CD8 T cells were observed polarizing granzyme B, TCR β , and CD8 protein consistent with activation and the formation of immune synapses.^{23,24} We have also determined that antiviral CD8 T cell morphological phenotypes are associated with motility.

CD8 T cells have been reported to use granzyme B to control HSV infection within sensory neuron cultures without inducing apoptosis.²⁵ To determine the mechanism by which CD8 T cells control TMEV infection and prevent demyelinating disease, we examined the molecular interaction between adoptively transferred GFP+ CD8+ lymphocytes and virus infected neurons. *In vivo* polarization

Figure 3. CD8 T cells form immune synapses with target neurons. Adoptively transferred GFP+ cells were found in close proximity to (A) TMEV-infected neurons and their processes. Shown in B is a round GFP+ lymphocyte without CD3 polarization (C) against a TMEV-infected neuronal body (D). These images are merged in E. For A–E, results are representative of four mice receiving adoptive transfer as defined in Figure 1. Both CD8 protein (F and J) and T cell receptor (G and K) polarized toward neurons (H and L) are highly indicative of an immune synapse. Merged images are shown (I and M). Enhanced CD8 expression (N) and granzyme B polarization (O) was present at the point of tight apposition between two CD8 T cells and a TMEV-infected neuron (P) and the merged images (Q). For F–Q, results are representative of an analysis of eight mice. All microscopy was performed on hippocampus. Scale bars: 50 μ m (A, left panel); 10 μ m (A, right panel); 10 μ m (B–Q).

Figure 4. Neurons have reduced surface H-2D^b class I molecules compared to lymphocytes. **A** and **E**: NeuN-positive neurons did not express high levels of H-2D^b class I molecule. By comparison, CD8⁺ cells directly adjacent to neurons (**B** and **F**) had high levels of class I molecule expression (**C** and **G**), and the merged images are shown in **D** and **H**. Results are representative of an analysis of hippocampal regions in four mice. Scale bars=10 μ m.

of CD3 protein against target neurons was not observed in these studies. This is an intriguing result, as both *in vivo* and *in vitro* studies have documented the presence of CD3 at immune synapses.^{26,27} In contrast, we determined that cellular engagement with neurons among CD8⁺ T cells was characterized by the polarization of CD8 protein, granzyme B, and TCR β . These observations are consistent with the previous analysis of CD8 T cell engagement with adenovirus-infected astrocytes.²³ Whether utilization of granzyme B by CD8 T cells contributes to the degenerative change observed in target neurons is currently under investigation, as TMEV in-

fection alone can result in neuronal death.²⁸ These studies also indicate that CD8 T cells have the capacity to simultaneously engage multiple targets *in vivo* as has been reported previously.²⁹

Consistent with previous reports, we did not observe high levels of MHC class I protein on the surface of neurons.^{1,15,30} However, these studies determined that T cell receptor could polarize toward neurons regardless of undetectable class I expression. While it could be inferred that class I is not expressed by neurons, an alternative possibility is that neurons express class I at a level below detection by immunohistochemistry. It is well doc-

Figure 5. Quantification of CD8 protein polarization toward neurons. The luminescent intensity of CD8 protein was higher near points of engagement among CD8 T cells engaging target neurons (**A** and **B**). Shown in **C** is a CD8 T cell polarizing CD8 protein toward two separate neurons simultaneously. Note that **arrows A, B, and C** in the microscopy images correspond to peaks of luminescent intensity [I]. Results are representative of an analysis of tissue slices that include striatum, hypothalamus, and cortex in eight TMEV-infected mice. Scale bars = 10 μ m.

Figure 6. Adoptively transferred CD8 T cells are morphologically diverse. Distinct morphological phenotypes were found among adoptively transferred cells. Round (**A** and **D**) and TCEP-presenting GFP+ cells (**E** and **M**) express CD3 (**B** and **F**) or CD8 protein (**J** and **N**). Neurons are distinguished by TMEV (**C** and **G**) or β III tubulin staining (**K** and **O**). The merged images are shown in (**D**, **H**, **L**, and **P**). Both round (**Q**) and TCEP-presenting CD8 T cells (**U**) were activated as indicated by granzyme B expression (**R** and **V**). GFAP staining is shown in **S** and **W** as a reference. Merged images are shown in **T** and **X**. Results are representative of an analysis of hippocampal regions in eight mice. Scale bars = 10 μ m.

Figure 7. Morphology and TCEP formation is consistent with movement as demonstrated by live tissue imaging. Adoptively transferred GFP+ cells (A) expressed CD18 (B), a component of the LFA-1 adhesion molecule complex, on the TCEP ($n = 4$ animals). In addition, adoptively transferred GFP+ CD8 T cells were assessed for mobility through hippocampal slices using live tissue imaging. Various morphologies were identified among GFP+ cells. C: Cells with round morphologies did not demonstrate movement or formation of projections for a period of 60 minutes (still frames from Supplemental Movie 1, at <http://ajp.amjpatbol.org>). D: GFP+ cells that were actively moving presented with a segmented morphology and traveled through tissue at a rate of approximately 1–2 $\mu\text{m}/\text{min}$ (still frames from Supplemental Movie 2, at <http://ajp.amjpatbol.org>). E: GFP+ cells were capable of rapid formation of TCEPs within 2 minutes that extend two cell lengths (still frames from Supplemental Movie 3, at <http://ajp.amjpatbol.org>). This cell rapidly extended and contracted these projections while remaining largely stationary over the time course observed. (Asterisks denote a relative point of origin in the tissue slice.) Movies depicted in C–E are representative of an analysis of two mice receiving adoptive transfer of CD8+GFP+ splenocytes. Scale bars: 10 μm (A); 75 μm (C and D); 25 μm (E).

umented that cellular targets of T cells with greatly reduced MHC molecule expression retain the capacity to form synapses.^{31,32} Therefore, though not expressed at the levels observed on CNS infiltrating lymphocytes, MHC class I molecules may be abundant enough on neurons to accommodate immune synapse formation.

The observation that CD8 T cells interact with neurons during TMEV infection is intriguing because these cells have been demonstrated to play a role in neuronal damage in both animal models and human disease conditions. Genetic disruption or suppression of CD8 T cell populations result in the preservation of axons and motor ability in chronically demyelinating, TMEV infected animals.^{15,33,34} Recently, we determined that CD8 and perforin-deficient mice had diminished capacity to develop

T1 hypointensities in TMEV infection as visible by MRI.³⁵ Additional MR spectroscopy analysis on the brains of these mice revealed that these T1 hypointensities are likely the result of axonal loss.³⁵ Furthermore, analysis of human MS lesions has correlated the presence of CD8 T cell infiltration with axonal damage.³⁶ Other human diseases such as Rasmussen encephalitis and progressive multifocal leukoencephalopathy also link CD8 T cells to neuronal pathology.^{37,38} Data presented in these studies demonstrate that direct engagement of neurons by CD8 T cells is a potential mechanism by which axonal and neuronal damage could occur in neuroinflammatory diseases. Therefore, analysis of this interaction in models of progressive demyelinating disease warrants further investigation.

Studies within the lymphoid compartments have observed unusual morphologies among “resting” lymphocytes. T lymphocytes up to five times in length as compared to width were reported.³⁹ These morphologies, previously only observed in lymph nodes, were readily apparent among CNS infiltrating antiviral CD8 T cells in our studies. In some instances, TCEP-presenting CD8 T cells had a total length of up to 45 μm , approximately five times the typical diameter of a lymphocyte. Large TCEP-presenting cells were also readily observed navigating through tissue. Immunohistochemistry and confocal microscopy revealed that the cytoplasm in large diameter TCEPs was often densely packed with granzyme B. Meanwhile, smaller diameter TCEPs contained very little granzyme B. Morphologically, these smaller TCEPs were similar to the rapidly extending and contracting structures observed in live tissue imaging. Given their dynamic nature, smaller TCEPs may be used to sample the microenvironment surrounding the cell. Also of interest, CD18, a component of the LFA-1 adhesion molecule, was observed on the tip of smaller TCEPs. This is suggestive that smaller TCEPs could contribute to CD8 T cells becoming motile.

With advances in two photon microscopy, the motility of both naïve and antigen-challenged T lymphocytes within lymphoid compartments are now being studied.^{39–41} Specifically, the motility of myelin basic protein-specific CD4 T lymphocytes in the mouse spinal cord has been analyzed.⁴¹ Kawakami et al determined that autoimmune CD4 T cells undergo various morphological changes and move at speeds up to 25 $\mu\text{m}/\text{min}$. “Resting” lymphocytes within lymphoid compartments move at an average speed of 10–12 $\mu\text{m}/\text{min}$.⁴² Meanwhile inflammatory conditions markedly reduced lymphocyte motility in the lymph node, potentially due to their engagement with resident antigen presenting cells.⁴² Here we demonstrate that brain infiltrating CD8 T cells have movement consistent with that analyzed in the lymph node, using a “stop-and-go burst pattern” of movement.³⁹ However, virus-specific CD8 T cells analyzed in these studies moved at greatly reduced speeds as compared with previous studies of lymphocytes imaged in the lymph node. The maximum speed observed among our brain infiltrating GFP+ cells was approximately 1–2 $\mu\text{m}/\text{min}$. These reduced speeds among brain infiltrating lymphocytes may be organ specific and/or the result of antigen presentation within the CNS during TMEV infection.

It has been suggested that observations of immobile T lymphocytes within lymphoid organs are due to improper methodology which ablates cytokine gradients necessary for movement.⁴³ However, in this analysis we observed both round and TCEP-forming CD8 T cells in high frequencies within the CNS. Both of these phenotypes were often observed in the same location in the brain, strongly reducing the likelihood of morphological changes in response to the loss of a cytokine/chemokine gradient. Therefore, the immobility of adoptively transferred CD8 T cells observed in these studies may imply that these cells have recognized an appropriate target and are undergoing sustained engagement. Such immobile behavior has been observed in “tethered” encephalitic

CD4 T cells infiltrating the spinal cord after antigenic challenge.⁴¹

We have previously published in several manuscripts that TMEV-infected C57BL/6 mice will mount a very focused CNS infiltrating D^b:VP2_{121–130} epitope specific CD8 T cell response naturally. The frequency of CD8 T cells recognizing this epitope ranges from 50 to 70% of total CD8+ cells.^{13,15,19} Among the remaining CD8 T cells that are not D^b:VP2_{121–130} epitope specific, roughly two thirds of these D^b:VP2_{121–130} tetramer negative cells express apoptotic markers and therefore appear to be dying (data not shown). The remaining viable D^b:VP2_{121–130} tetramer negative CD8+ cells remain unidentified. It is possible that these viable tetramer negative CD8+ cells observed by flow cytometry are a distinct lymphocyte lineage that does not recognize the D^b:VP2_{121–130} epitope. Lyman et al have defined the minor epitopes, D^b:VP2_{165–173} and D^b:VP3_{110–120}, that are recognized by CNS infiltrating CD8+ T cells during acute TMEV infection.⁴⁴ Alternatively, these cells could include CD8+ dendritic cells or nontraditional CD8+ lymphocytes. We reason that these tetramer negative cells likely originate from a different organ than the spleen, as the majority of CD8+ donor splenocytes develop into traditional D^b:VP2_{121–130} epitope specific CD8 T cells (Figure 1).

In this report, we have analyzed the cellular movement and molecular interactions of antiviral CD8 T cells within the CNS. Using these methods, we have determined that during acute TMEV infection, virus-specific CD8 T cells have the capacity to engage neurons expressing viral protein. These observations provide insight into the potential mechanism by which virus is cleared from TMEV-resistant strains of mice ultimately protecting against the demyelinating syndrome. These studies also demonstrate the capacity of CD8 T cells to polarize effector molecules against neurons, a process implied to occur in MS lesions.^{36,45} The engagement of CD8 T cells with neurons may therefore protect against demyelination syndrome but may also mediate neuronal cell death in neurological diseases characterized by infiltration of these lymphocytes.

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