UNIVERSITY OF CINCINNATI

Date: 21-Aug-2009

I, Jeremiah McDole, hereby submit this original work as part of the requirements for the degree of:

Doctor of Philosophy

in Neuroscience/Medical Science Scholars Interdisciplinary

It is entitled:

CNS-infiltrating CD8 T cells become virus-specific and engage neurons during TMEV infection

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CNS-infiltrating CD8 T cells become virus-specific and engage neurons during TMEV infection

A dissertation submitted to the Graduate School of the University of Cincinnati in partial fulfillment of the requirements for the degree of Doctor of Philosophy (Ph.D.) in the Graduate Program in Neuroscience of the College of Medicine by

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August 2009

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Abstract

Compelling research has linked CD8 T cells to neuronal injury, a pathological hallmark of multiple sclerosis. The epitopes recognized by CD8 T cells in MS patients remains unknown. Defining these epitopes is of potentially critical importance to developing new therapeutics for MS, an aspect of which has been explored by our lab. In previous studies using the established Theiler’s murine encephalomyelitis virus (TMEV) model of MS we demonstrated that expansion of an immunodominant population of CD8 T cells could be inhibited by the administration of the epitope peptide that they recognized. Inhibiting the expansion of this CD8 T cell population resulted in reduced levels of neuronal injury and sparing of motor ability. In work presented here we have developed an unbiased, molecular approach for determining CD8 T cell epitopes using the TMEV model. Such an approach could be potentially translated from this animal model to MS, possibly contributing to future therapeutics. Further, defining the putative mechanism utilized by CD8 T cells to perpetuate neuronal pathology in diseases such as Rasmussen’s encephalomyelitis, neurological neoplastic disorders and MS remains a critical topic of research. Defining this mechanism could have important ramifications for the development of new treatments for these conditions. In studies presented here using the TMEV model we demonstrate evidence that strongly suggests CD8 T cells form traditional immune synapses with neurons. Further, we demonstrate that CD8 T cells also polarize granzyme B, an effector molecule that can potentially induce apoptosis, towards target neurons. Taken together this evidence provides a potential mechanism utilized by CD8 T cells to perpetuate neuronal pathology. Lastly, we demonstrate in these studies that morphological changes observed in CD8 T cells are
associated with movement through CNS tissue. The motility patterns of these CNS infiltrating CD8 T cells was similar to the movement observed in other studies examining T cells within lymph nodes. Such observations may suggest that a common cellular mechanism controls morphological changes and motility among CD8 T cells regardless of the organ within which they are derived.
Acknowledgments

Aaron J. Johnson Just when things start to go right its time to go. You pushed me continuously over the years and it was exactly what I needed. For me, it felt like a rough start. I did not know much and external circumstances seemed to divide my focus down a hundred random paths but with your help I made it. You managed to find the positive side among so many failed experiments and that was important for me. You allowed me a great deal of independence and I think it paid off. I can honestly say that there is not another lab I would rather have been a part of as a graduate student.

Istvan Pirko Thank you for all of the advice and insight. You provided a million laughs for me and the rest of the lab and so many times it was sorely needed. You have been encouraging and patient. Any student would be fortunate to have you as an advisor.

Thesis committee Kim Seroogy, you have done so much for this program and remain a constant advocate for its students. Thank you for agreeing to chair my committee and all of your excellent advice. Steve Danzer, thank you for providing so much guidance for my microscopy projects. I hope you had as much fun finding those crazy cells as I did.

David Hildeman, your ideas were always nothing short of brilliant. Thank you for your patience when dealing with my lack of immunology background and thanks for helping me find the lymph nodes.

Lab members Georgette Suidan, you have been a lot of fun. I really enjoyed our talks, they were very therapeutic. Enjoy Harvard I look forward to seeing you again. Yi Chen, you are the duct tape in our lab, we’re helpless without you. To everyone else both past and present, thank you for all of your help and all of the conversations about nothing and everything.
Neuroscience program I truly consider myself fortunate to have been a part of this graduate program. Many of the individual members have helped keep me going. Dr. Herman, we are all grateful to have you as head of our program. You have tirelessly led us through stormy waters and continue to do so. I am especially grateful for your sense of humor, I am sure that I tested it extensively. Deb Cummins, you keep this program moving forward, without you we would be lost. Jon Dickerson, hundreds of lunch time conversations… could 5 years really have gone so quickly? Ann Hemmerle, I probably would not have made it on time to a single class my first year without you. Matt Loftspring, I will miss our hair-brained collaborations. Erin Boespflug, I am glad you did not take my advice. To all of the students and faculty who have given invaluable guidance along my journal, thank you.

Neurology department Thank you for all of your hard work and assistance throughout the years.

Family and friends Thank you for helping me stay grounded and keeping my life interesting.

…and my beloved wife, the single greatest influence in my life.
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<th>Description</th>
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<tr>
<td>LFA-1</td>
<td>Lymphocyte function associated antigen 1</td>
</tr>
<tr>
<td>CD3</td>
<td>Cluster of differentiation 3</td>
</tr>
<tr>
<td>CD4</td>
<td>Cluster of differentiation 4</td>
</tr>
<tr>
<td>CD8</td>
<td>Cluster of differentiation 8</td>
</tr>
<tr>
<td>CD18</td>
<td>Cluster of differentiation 18</td>
</tr>
<tr>
<td>TMEV</td>
<td>Theiler’s murine encephalomyelitis virus</td>
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<tr>
<td>TCEP</td>
<td>T cell extended process</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility</td>
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<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>GFAP</td>
<td>glial fibrillary activation protein</td>
</tr>
<tr>
<td>NeuN</td>
<td>Neuronal nuclei</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TCRβ</td>
<td>T cell receptor beta</td>
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<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
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<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescent activated cell sorting</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>GzmB</td>
<td>Granzyme B</td>
</tr>
<tr>
<td>EAE</td>
<td>Experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebral spinal fluid</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>MBP</td>
<td>Myelin basic protein</td>
</tr>
<tr>
<td>APP</td>
<td>Amyloid precursor protein</td>
</tr>
<tr>
<td>MOG</td>
<td>Myelin oligodendrocyte glycoprotein</td>
</tr>
<tr>
<td>NAA</td>
<td>N-acetylaspartate</td>
</tr>
<tr>
<td>β2m</td>
<td>Beta-2-microglobulin</td>
</tr>
<tr>
<td>MRS</td>
<td>Magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe combined immunodeficiency</td>
</tr>
<tr>
<td>RAG</td>
<td>Recombinase activating gene</td>
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Chapter one

Introduction: *CD8 T cells in lesion formation and axonal dysfunction in multiple sclerosis***

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Multiple sclerosis (MS) is the most common demyelinating disease of the human central nervous system (CNS). MS is typically first diagnosed in individuals between 20 and 40 years of age. Approximately, 80 percent of patients present with relapsing-remitting MS (43). This condition typically starts with clinical signs such as sensory disturbances, unilateral optic neuritis, diplopia, limb weakness, clumsiness, gait ataxia and bladder and bowel symptoms (43). Aphasia, apraxia, recurrent seizures, visual-field loss may also be present. Additionally, fatigue may accompany increases in physiological body temperature in these patients (43). In this condition, clinical symptoms surface over the course of several days, stabilize, and then improve. Pathological changes also aid in diagnosing relapsing-remitting MS. Multifocal lesions can be visualized on MRI. These lesions consist of well defined areas of myelin loss and the formation of astrocytic scars (43). Axonal injury is also present which is thought to result in progressive neurological loss of function (43). MS lesions are concentrated within the periventricular white matter, brain stem, cerebellum, and spinal cord white matter. These lesions may spread in time with the accompaniment of new lesion formation (43). Of all MS patients, 70 percent will have the relapse-remitting condition accompanied by secondary progression (57). These patients often have persistent signs of central nervous system dysfunction after a relapse, and the disease may progress between relapses. As the disease progresses cognitive impairment, depression, dysarthria, dysphasia, vertigo, progressive quadriaparesis, sensory loss, ataxic temors, pain, sexual dysfunction and spasticity often become severe (43). In spite of the symptoms, life expectancy may be shortened only slightly (43). Drug-based treatment of
MS is very limited. Acute administration of corticosteroids is often used to shorten the recovery time from clinically severe relapses (43). Other drugs such as interferon beta-1b, interferon beta-1a and glatiramer acetate are often prescribed over the long-term course of the disease. All of these drugs reduce the occurrence of new lesions and may reduce the frequency of relapses but none have shown efficacy at reducing long-term clinical decline (23). Some relapsing-remitting patients are considered to have benign MS. This condition occurs in approximately 10 percent of all MS patients (43). These patients may only have mild, non-progressive symptoms or remain completely asymptomatic.

Approximately, 20 percent of patients present with primary progressive MS. These patients often present with slowly evolving upper-motor-neuron syndrome of the legs (43). As the disease progresses quadriplegia, cognitive decline, visual loss, brain-stem syndromes, and cerebellar, bowel, bladder and sexual dysfunction can develop (43). This condition is characterized by a gradually progressive clinical decline. Currently, there are no proven therapies for primary progressive MS (43).

While the etiology of MS is unclear it is accepted to be an immune-mediated disease. Infiltrating immune cell populations are thought to be responsible for the observed tissue damage found in MS lesions and throughout the CNS of patients (43). All major immune cell populations, including macrophages, B cells, CD4 T cells, NK cells and CD8 T cells have been implicated as mediators of the inflammatory cascade found in MS. Among these cell types CD8 T cells are particularly well suited to play a detrimental role in MS lesions due to their ability to perform cell-killing, effector functions as well as their dominant presence in MS lesions.
The function of CD8 T cells

Typically, CD8 T cells assist in the clearance of viruses from an infected organism. Viruses replicate inside of the cells that they infect. During replication some of the viral protein is degraded into peptide fragments. These fragments are then loaded onto MHC class I molecules. This combination of MHC class I and viral peptide is then expressed on the surface of the virus infected cell. CD8 T cells express T cell receptors (TCRs) that have undergone gene rearrangements that make their specificity highly variable. In spite of this high variability, these TCRs can only interact with a limited number of combinations of MHC class I:peptide complexes. If the TCR of a CD8 T cell is specific for the MHC class I/viral peptide combination from an infected cell, it has the capacity to kill that cell through cell-cell engagement.

There are many hypotheses to explain why CD8 T cells could potentially create pathology within MS patients. One hypothesis puts forward that some undefined pathogen, such as a virus, is present within the CNS of MS patients (20). Unable to clear the pathogen, CD8 T cells continuously kill infected cells rendering gross tissue destruction. Another hypothesis, known as molecular mimicry, suggests that overlap occurs between the syngenic peptides presented by MHC class I molecules under non-disease conditions and pathogen peptides presented during a state of disease (59). Syngenic peptide is ubiquitously expressed by class I molecules on all nucleated cells under non-disease circumstances. Autoreactive CD8 T cells that might recognize this syngenic peptide are selected against during lymphocytic development, leaving a population of CD8 T cells that only recognize foreign peptide. However, due to the
limited repertoire of possible combinations, overlap between syngenic and foreign peptide it believed to occur. Therefore, a CD8 T cell primed against a pathogen could recognize syngenic peptide as foreign leading to the engagement and death of a healthy cell. The last hypothesis is known as epitope spread (30). According to this hypothesis, syngenic peptide comes to be recognized as foreign due to an initial event in which tissue damage takes place. This initial tissue damage could be the result of a CNS virus infection, for instance. In this process, developing CD8 T cells are primed against peptides derived from the damaged syngenic tissue. These cells could then recognize a large repertoire of syngenic peptides as foreign, potentially causing a wide-spread autoimmune response resulting in the death of healthy cells.

Extensive work in tissue obtained from MS patients has provided insight into how the immune system may be perpetuating disease. This work has provided convincing evidence that CD8 T cells play a potentially crucial role in MS. These studies demonstrate that CD8 T cells are the most numerous lymphocytic cell type in MS lesions, are clonally expanded in patients, and are correlated with the presence of neuronal injury.

**CD8 T cells are clonally expanded and potential effectors in MS**

CD8 T cells are potential candidates for effector cells in MS for two important reasons. First, CD8 T cells express molecules that could mediate tissue destruction, including Fas ligand, granzyme B and perforin(19, 31, 42). CD8 T cells have been shown to be capable of killing multiple CNS cell subtypes in vitro, including oligodendrocytes and neurons (49, 53). CD8 T cells have been detected in close proximity to demyelinated axons within MS brain tissue (42). These CD8 T cells expressed granzyme B, a cell death-inducing molecule and activation marker. Additionally, the cytotoxic granules of
these CD8 T cells appeared to be polarized in the direction of the neurons suggesting cytotoxic attack (42). This is feasible because neurons within MS lesions have been shown to express class I molecules therefore making them prime targets for infiltrating cytotoxic T cells (24).

Second, clonally expanded CD8 T cells are present within MS patients. CD8 T cells with focused T cell receptor (TCR) specificity have been observed in MS lesions (2). This suggests that the CD8 T cells found in MS lesions are focused on a common yet undefined antigen. This also indicates that CD8 T cells may be actively performing an effector function potentially detrimental to CNS tissue. Quantitative studies have found dominant populations of CD8 T cells present in MS lesions (4, 22, 58).

Babbe et al. demonstrated that large populations of clonally expanded CD8 T cells can be found in both active and inactive MS lesions based on studies of biopsy material from two MS patients (2). This study also sought to identify the antigens recognized by the TCRs expressed by CD8 T cells located within lesions. In the biopsy materials obtained from the first MS case, 65% of the CD8 T cells sampled represented clonally expanded populations. Interestingly, one clonally expanded subset of CD8 T cells comprised 35% of the total cells from all sampled lesions. In the other biopsy case, only one lesion was available for analysis. However, this single lesion demonstrated extensive clonal expansion of the CD8 T cell populations. It is important to note that the clonally expanded CD8 T cells found in this lesion were also present in the peripheral blood. This indicates migration of clonally expanded CD8 T cells between CNS tissue and blood of MS patients.
Similar to the brain, CSF harbors CD8 T cells with clonal expansion (27, 50). Skulina et al. examined the TCR repertoire from tissue sampled from brain biopsies, blood and CSF of two MS patients. One of the brain biopsy samples had been previously examined using microdissection and single-cell PCR to identify infiltrating T cell populations (2). Utilizing complementary determining region 3 spectratyping, the presence of multiple clonally expanded CD8 T cell populations were confirmed in brain, CSF and blood. Interestingly, some of the brain-infiltrating clonally expanded CD8 T-cells were present in CSF or blood for over 5 years, suggesting ongoing exposure to a persistent antigen and the acquisition of memory T cells specific for a particular antigen.

**CD8 T cell epitopes in MS are not known**

In light of their potential effector role and clonal expansion within MS patients, the peptide epitopes for which CD8 T cells are specific must be identified. Discovering these epitopes could potentially aid in our understanding of the disease mechanism of MS. If we could distinguish whether these epitopes were derived from syngenic or pathogenic peptides could help us determine if MS is an autoimmune process or pathogen driven condition. Further, discovery of CD8 T cell epitopes could aid in the development of future therapeutics. Our lab has successfully used the administration of peptide epitopes to inhibit the expansion of pathogenic CD8 T cell populations within animals induced with an MS-like condition (28). Inhibition of these CD8 T cell populations resulted in the therapeutic sparing of motor ability among these animals with MS-like symptoms. Similarly, if the epitope specificity of potentially pathogenic CD8 T cells within MS patients could be identified administration of peptide for which they are specific could also act to inhibit these cells. Inhibition of pathogenic CD8 T cells could
lead to reduced pathology and reduced clinical symptoms as was found in our animal model.

CD8 T cell epitopes in MS has been the subject of much work (25). It has long been hypothesized that some component of oligodendrocytes or the myelin sheath they provide for axons would be the epitope target for CD8 T cells. Much of this reasoning is based on the experimental autoimmune encephalomyelitis (EAE) animal model of MS (26, 51). In this model, autoimmune T cells specific for myelin sheath peptides create demyelinating lesions along the spinal cord of animals, a pathological symptom of MS. While T cell epitope specificity is well defined in the EAE model, there is little data regarding the epitope specificity of T cells in human MS patients. Antibodies isolated from the cerebrospinal fluid of MS patients were found not to be specific for certain myelin epitopes (45). Whether, this will serve as a clue to the epitopes for which CD8 T cells are specific remains to be seen. Therefore, the identification of potential CD8 T cell epitopes in MS patients remains an illusive, yet critical component for future treatments.

**Neuronal pathology in MS**

Research of MS has mostly focused on demyelination. However, the absolute effects of ‘pure’ demyelination in the absence of axonal and/or neuronal pathology on disability may be rather limited. In addition to demyelination, axonal and neuronal dysfunction is now considered an integral component of MS pathology that leads to clinical disability (7, 8, 11). There is experimental evidence suggesting that despite extensive demyelination, in the absence of axonal or neuronal pathology, clinically evident disability may not develop (47). This is potentially due to compensatory mechanisms induced by neurons to reduce the effects of myelin sheath loss.
channel redistribution can occur in both human and animal neurons after loss of the myelin sheath (9, 37). Increased numbers of mitochondria in demyelinated axons have also been observed (1).

Many studies have demonstrated the ubiquitous nature of axonal injury in MS. Direct methods to demonstrate axonal transection and injury now exist. These techniques exploit the presence of axonal spheroids or terminal ovoids, reductions in neurofilament and accumulations of amyloid precursor protein (APP) in MS patient tissue (13, 33, 54). In addition to histological analysis, magnetic resonance spectroscopy (MRS) has also been used in many studies to explore axonal loss and its role in MS (14-16, 18). It is particularly useful because it allows the assessment of axonal dysfunction and loss in vivo. One of the metabolites detected on proton MRS spectra, N-acetylaspartate (NAA), has been shown to be highly localized to the axons and neuronal bodies of the mature human brain. However, whether reduction of NAA indicates total axonal loss or only injury is a matter of contention (39). A 2001 study by De Stefano et al. used this technique to examine neuronal damage in 88 MS patients (17). Of the patients studied, a significant decrease in NAA signal was found among individuals with MS as compared with healthy controls, indicating neuronal loss. Interestingly, decreases in NAA signal were found in the earliest stages of the disease as well as in the normal appearing white matter, suggesting significant axonal dysfunction or damage in MS (21).

**Neuronal pathology correlates with clinical disability in MS**

Neuronal injury robustly correlates with the clinical outcome of MS patients. Losseff et al. described a highly reproducible MRI technique that allowed accurate quantification of spinal cord atrophy (32). This study found that atrophy, including tissue
loss as a result of axonal dropout, strongly correlated with clinical disability. Many studies since have confirmed these observations in both cerebrum and spinal cord (34, 40, 56). Further studies continue to demonstrate that neuronal damage correlates with the clinical course of MS (6, 12).

**CD8 T cells as effectors of neuronal pathology in MS**

Histological evidence points to CD8 T cells as effector cells in axonal loss. A study by Bitsch et al. included 88 biopsy samples containing 109 MS lesions from 42 patients (5). This study used accumulation of APP as a marker of axonal damage. It was found that an increased frequency of CD8 T cells significantly correlated with the presence of increased APP within neuronal axons. Studies examining other inflammatory diseases of the CNS such as paraneoplastic disorders and Rasmussen’s encephalitis have presented additional evidence to support the hypothesis that CD8 T cells contribute to neuronal pathology in patients (3, 52).

**Direct vs. indirect engagement of neurons by CD8 T cells**

There is mounting evidence to support the idea that CD8 T cells contribute to neuronal pathology in MS. Two mechanisms have been hypothesized by which CD8 T cells may injure or kill neurons. The direct engagement hypothesis puts forward that CD8 T cells can directly engage a neuron via an MHC class I:peptide/TCR interaction. This direct engagement could then lead to neuronal injury or apoptosis (35). In support of this hypothesis, some investigators have demonstrated functional expression of MHC class I on neurons (10). However, many studies have been unable to show robust neuronal staining of MHC class I, a molecule believed to be necessary for direct CD8 T cell engagement (29). Further, most glial cell types have been shown to express class I
and are susceptible to lytic attack by CD8 T cells (42). This evidence has led to the more popular indirect engagement hypothesis of neuronal injury by CD8 T cells (35). This hypothesis puts forward that CD8 T cells engage and potentially kill glial cells and in the process create an unhealthy environment for neighboring neurons. This damaging environment could then lead to neuronal injury or death.

**Animal models of MS**

It is important to study CD8 T cell engagement of CNS cell types in order to establish this lymphocyte’s potential role in perpetuating neuronal pathology in MS. However, studying these interactions in MS is difficult due to the rarity of human tissue and the lack of experimental tools available to researchers. Therefore, animal models are employed. The two major animal models of MS are EAE and Theiler’s murine encephalomyelitis virus (TMEV) infection (41). EAE is a well established model of autoimmune demyelination. In this model, mice are immunized against myelin, myelin components or related synthetic peptides. These synthetic peptides include myelin oligodendrocyte glycoprotein (MOG), myelin basic protein (MBP), and myelin proteolipid protein (PLP) (41). The autoimmune inflammatory response to myelin or myelin peptides that follows immunization results in demyelination. Clinical disease is usually relatively mild and most often manifests as a limp tail (41). More severe disease may cause weakness in one or both hind limbs in mice. Clinical symptoms usually resolve relatively quickly, leaving the animal with no long-term motor dysfunction. Rarely, some animals incur a relapse-remitting disease in which the initial bout of motor dysfunction and recovery is followed by a less severe relapse of muscle weakness (46). Myelin-reactive CD4 T cells mediate the inflammatory demyelination process which
leads to muscle weakness. CD4 T cells dominate EAE lesions which reside primarily in the spinal cord of immunized animals. Traditional EAE models do not have a defined role for CD8 T cells (41).

Due to the relative lack of a CD8 T cell component in the EAE model, the intracranial TMEV infection model of MS was utilized for studies presented in this work. There are strains of mice susceptible and resistant to chronic TMEV-induced disease (44). When infected with TMEV, susceptible stains of mice incur a biphasic disease, consisting of early acute disease followed by late chronic demyelinating disease. These animals do not clear virus from the CNS. In contrast, resistant animals incur only acute disease and ultimately clear virus from the CNS.

Early acute disease occurs 3 to 12 days post-infection and resembles polioencephalomyelitis (38). It is associated with various degrees of CNS-infiltrating CD4 T cells, CD8 T cells, macrophages and to a lesser extent B cells and plasma cells (44). During acute disease, virus primarily infects cells of the hippocampus, striatum, and cortex. Various degrees of neuronal death are also present in early acute disease. Susceptible animals then incur late chronic demyelinating disease approximately 30 to 40 days post-infection with virus infection found primarily within the spinal cord (38). Mononuclear cellular infiltrates are present within the spinal cord and consist mostly of CD4 T cells, CD8 T cells and macrophages (44). This disease leads to extensive demyelinating lesions in the white matter of the spinal cord, spinal cord atrophy and axonal loss. Clinical symptoms include disruption of motor coordination, limb paralysis, spasticity, ataxia, and incontinence.
Many immune cell types have been shown to be involved in both maintaining resistance to TMEV infection as well as perpetuating disease in susceptible strains of mice. However, susceptibility to chronic TMEV-induced disease has been shown to be MHC class I haplotype dependent (48). This provides strong genetic evidence that CD8 T cells play a crucial role in controlling TMEV infection because they are functionally MHC class I restricted. Studies have demonstrated that genetically knocking out CD8 T cells in resistance strains of mice results in the induction of susceptibility in these animals (36). Conversely, one study showed that by inhibiting the expansion of CD8 T cells in a susceptible strain of mouse resulted in the sparing of motor ability in these animals (28).

A model to study CD8 T cell interactions with CNS cell types

The TMEV infection model of MS has both a CD8 T cell component and the occurrence of neuronal pathology potentially providing an excellent model in which to study CD8 T cell interactions with CNS cell types (44). The CD8 T cell response to TMEV infection in the C57BL/6 mouse is well established. It has been demonstrated that high levels of CD8 T cells infiltrate the CNS of these animals upon TMEV infection. Further, approximately 70 percent of these infiltrating CD8 T cells are specific for a single TMEV epitope peptide, VP2\textsubscript{121-130} (28). Although, the C57BL/6 mouse strain is resistant to chronic TMEV-induced demyelinating disease, extensive neuronal apoptosis has been shown to occur (55). Taken together, TMEV infection of the C57BL/6 mouse provides a model in which high numbers of virus specific CD8 T cells infiltrate the CNS and are provided the opportunity to interact with virus infected CNS cell types in an antigen specific manner, potentially contributing to neuronal pathology. Because of these benefits this model was ultimately used for the studies presented in this work.
Hypothesis and rationale

The capacity for CD8 T cells to directly engage neurons is poorly understood. Further, there is no in vivo evidence demonstrating that a CD8 T cell can directly engage a neuron in an antigen specific manner. TMEV infection of the C57BL/6 mouse was utilized to explore these topics. Our working hypothesis for this research is that CD8 T cells can directly engage neurons and do so in an antigen specific manner. Our rationale for performing this research is to establish whether CD8 T cells can directly engage neurons. Such work is potentially translatable to human conditions such as MS, Rasmussen’s encephalomyelitis, and paraneoplastic disorders. In all of these diseases CD8 T cells may be linked to neuronal pathology (3, 52). Although, a strain of mouse resistant to chronic TMEV-induced disease is utilized neuronal pathology does occur and it provides an excellent model to study CD8 T cell interactions with CNS cell types. Further, a common mechanism of engagement may be shared between this animal model and human disease conditions.
References


Chapter two

Peripheral and CNS infiltrating CD8 T cells become specific for the VP2121-130 epitope under TMEV infection**

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Abstract

Defining the epitope specificity of CD8 T cells is an important goal in autoimmune and immune mediated disease research. We have developed a translational molecular approach to determine the epitope specificity of CD8 T cells using the Theiler’s murine encephalomyelitis virus (TMEV) model of multiple sclerosis (MS). TMEV-specific CD8 T cells were isolated from brains and spleens of 7-day TMEV-infected C57BL/6J mice and stimulated by Cos-7 cells that were co-transfected with expression vectors encoding the D^b class I molecule along with overlapping segments of the TMEV genome. Both brain-infiltrating and spleen-derived CD8 T cells expressed IFN-γ when Cos-7 cells were co-transfected with D^b class I molecule and the TMEV genomic segment that encoded the immunodominant TMEV epitope. This demonstrated that peripheral and brain-infiltrating CD8 T cell responses were focused on peptide epitope(s) encoded by the same region of the TMEV genome. We propose that a similar molecular approach could also be used to determine the antigen specificity of suppressor CD8 T cells by the measurement of transforming growth factor–β (TGF-β) production. In addition, with a randomly generated library and peripheral blood or isolated CSF CD8 T cells, this would be an effective method of predicting the epitope specificity of CD8 T cells in human inflammatory CNS diseases, in animal models of MS or other organ-specific inflammatory diseases with a protective or pathogenic role of CD8 T cells.
**Introduction**

The role of central nervous system (CNS)–infiltrating CD8 T cells is an active area of CNS inflammatory disease research. Studies have demonstrated that CD8 T cells are prevalent in multiple sclerosis (MS) lesions, outnumbering other lymphocytes at all stages of lesion development (1, 21). CD8 T cells have also been shown to be clonally expanded in lesions, cerebrospinal fluid (CSF), and peripheral blood, suggestive of a common epitope, or a restricted number of epitopes being recognized by this cell type (1, 24). In addition, it has been demonstrated that T cell receptor (TCR) sequences from clonally expanded CD8 T cells can persist for years indicating ongoing activation of memory CD8 T cell clones. Whether these epitope-specific CD8 T cells act as suppressors of inflammation or effectors of pathology remains to be determined.

A major hypothesis generated through human tissue and animal model studies provides evidence for a suppressor role of CD8 T cells. Work in human samples has relied on characterizing functional aspects of cultured CD8 T cells derived from the peripheral blood of MS patients. Findings from this work have suggested that a deficiency of suppressor function by CD8 T cells may be linked to MS pathologic conditions (27). The majority of suppressor CD8 T cell work has been performed in the experimental allergic encephalomyelitis (EAE) animal model of MS. In EAE, the absence of CD8 T cells results in the worsening of disease or susceptibility to secondary induction of EAE (8, 11, 16). Mechanistic studies have provided evidence that suppressor CD8 T cells act via T-cell receptor interaction with the Qa-1 molecule presented by activated CD4 T cells to cull inflammatory responses. Qa-1–independent mechanisms involving cytokine mediated suppression by CD8 T cells have also been proposed (6, 20).
Although not mutually exclusive from the suppressor CD8 T cell hypothesis, independent studies also support an effector role for this cell type in the pathology in MS patients. Studies in human tissue have demonstrated that all CNS cell types within an MS lesion have the capacity to express major histocompatibility complex (MHC) class I molecules and can potentially be targeted by CD8 T cell–mediated cytotoxicity (7). Also supporting a role for cytotoxicity by activated CD8 T cells is their close proximity to oligodendrocytes and demyelinated axons in brain tissue from MS patients. In EAE models, CD8 T cells have been shown to be sufficient to adoptively transfer disease (9, 25). In the Theiler murine encephalomyelitis (TMEV) model, many aspects of clinical disease, including axonal dysfunction, demyelination and motor dysfunction, have been linked to CD8 T cells (13, 19, 29, 31).

To determine the extent CD8 T cells acquire suppressor and effector functions, the epitope specificity of CD8 T cells in MS needs to be defined. Numerous approaches have been demonstrated to investigate the epitope specificity of CD8 T cells in TMEV infection, including the use of overlapping peptide libraries, molecular expression of TMEV proteins, stable transfected cell lines, and recombinant vaccinia virus vectors (2, 17, 28, 30). These approaches have unequivocally demonstrated the utility in defining CD8 T cell epitopes in TMEV infection. However, none of these previously developed systems are feasible approaches to define CD8 T cell epitopes in human MS in which there are logarithmically higher numbers of potential antigenic targets and the genomic sequence encoding these antigenic determinants are unknown. We therefore have developed an unbiased translational molecular approach to identify CD8 T cell epitopes. In this proof-of-principle study, we successfully used this approach to identify the region
of the TMEV genome that encodes the immunodominant epitope recognized by CNS-infiltrating CD8 T cells in the C57BL/6 mouse strain. Although this mouse strain is resistant to chronic demyelination, as it effectively clears the virus in the acute meningoencephalitis stage, it has been demonstrated that demyelination may occur in mice with C57BL/6 background by an intravenous injection of the immunodominant peptide on day 7, or by the use of mice deficient in interferon-γ receptor (22, 23). Furthermore, there is no theoretical limitation for this method to detect epitopes in demyelinating strains or to detect polyclonal CD8 T cell responses. In this study, CD8 T cells from the spleen were stimulated by the identical TMEV library segment as CD8 T cells isolated from the brain. This interesting observation strongly suggests a specific focus among CD8 T cells to a conserved immunodominant epitope, regardless of being isolated from brain or a peripheral lymphoid compartment. This molecular approach with a large organ-specific cDNA library derived from MS lesions, similarly to what has already been accomplished in autoimmune diabetes studies (32), has the potential to be adapted for clinical use with peripheral human CD8 T cells derived from the blood or the CSF as a noninvasive method to predict the epitope specificity of CNS-infiltrating CD8 T cells in MS patients.
Results

Generation of the TMEV library

To define the TMEV epitopes recognized by CD8 T cells directly isolated \textit{ex vivo} from brains and spleen, 14 segments approximately 560 base pairs in length and overlapping by approximately 30 base pairs were PCR amplified from the TMEV genome (Figure 1A). The overlap in this library enables a comprehensive analysis of all potential class I binding peptides encoded by the TMEV genome. Segment 15 encoded the L* protein, an alternate reading frame that has been noted to produce protein critical to TMEV infection (5). Each segment of the genome was inserted into the pcDNA 3.1 His A vector and sequenced (Figure 1B). The pcDNA 3.1 His A vector expresses well in mammalian cell lines ideally matched for transfecting Cos-7 cells. Individual wells of Cos-7 cells were then co-transfected with the D\textsuperscript{b} or K\textsuperscript{b} MHC class I molecule and a TMEV genomic segment. CD8 T cells isolated from the brain or spleen of a 7-day TMEV-infected mouse were cultured with co-transfected Cos-7 cells. Upon epitope recognition CD8 T cells expressed IFN-γ into culture media later detected by ELISA. Therefore, IFN-γ was expressed only in wells co-transfected with an MHC class I molecule/TMEV epitope recognized by an \textit{ex vivo} isolated CD8 T cell.

Efficient co-transfection of class I and GFP-expressing plasmid vectors in Cos-7 cells

Next we addressed the efficacy of co-transfection of class I molecules and GFP in Cos-7 cells. To accomplish this, Cos-7 cells were sham transfected or co-transfected with
Kb or Db and with GFP and analyzed by flow cytometry (Figure 2). Wells sham transfected with empty pcDNA 3.1 His A vector or co-transfected with Kb or Db class I molecule plus GFP were incubated for 48 hours. Cells were then resuspended from wells using cold EDTA PBS and stained with antibody specific for both Kb and Db. No expression of GFP, Kb, or Db was observed in sham-transfected Cos-7 cells (Figure 2A). In contrast, both Kb and Db had marked expression simultaneously with GFP. Approximately 21.7% of co-transfected Cos-7 cells expressed both Db and GFP (Figure 2B). Meanwhile, 24.9% of co-transfected Cos-7 cells expressed both Kb and GFP (Figure 2C). This experiment showed that a significant portion of Cos-7 cells could be co-transfected with class I molecule and a second plasmid encoding GFP, demonstrating the feasibility of this approach to stimulate ex vivo isolated CD8 T cells with TMEV genome segments.

**Recognition of TMEV-specific CD8 T cells derived from brain and spleen is restricted to the library fragment encoding the immunodominant epitope**

After establishing that Kb and Db class I molecules were expressed on the surface of transfected Cos-7 cells, the efficiency of antigen processing and presentation was tested. Cos-7 cells were co-transfected with Db and pcDNA 3.1 His A/VP2, which encodes the immunodominant VP2_{121–130} peptide (genomic base pairs 1867–1896) or Db plus an empty pcDNA 3.1 His A vector. The D^b:VP2_{121–130} complex has been established as the immunodominant epitope detected by brain-infiltrating CD8 T cells in C57BL/6 mouse strain when infected with TMEV (12). CD8+ T cells isolated from the brains of 7-day TMEV infected C57BL/6 mice were plated onto these transfected Cos-7 cells. A
higher signal for IFN-γ was detected by ELISA in wells co-transfected with D^b and pcDNA3.1 His A/VP2 than in wells transfected with D^b plus empty pcDNA 3.1 His A vector (Figure 3A). CNS-infiltrating CD8 T cells were also analyzed by flow-cytometric analysis. Consistent with previous reports, approximately 55% epitope dominance for D^b:VP2_{121-130} among CD8 T cells was observed (Figure 3B). This demonstrated that CD8 T cells isolated from the CNS could directly recognize the immunodominant D^b:VP2_{121-130} epitope presented by Cos-7 cells co-transfected with D^b plus pcDNA 3.1 His A/VP2.

We next wanted to determine the extent that CD8 T cells isolated from peripheral lymphoid compartments were focused on specific TMEV epitopes 1 week post–intracranial infection. Previous work involving the use of D^b:VP2_{121-130} tetramers determined that low numbers of D^b:VP2_{121-130} epitope-specific CD8+ T cells were observed in the spleen of 7-day TMEV-infected C57BL/6 mice (12). We therefore used the TMEV library approach to determine how focused the CD8 T-cell response was towards the immunodominant VP2_{121-130} peptide. Our molecular approach, unlike tetramer technology, would also determine whether there were epitopes other than D^b:VP2_{121-130} recognized by spleen-derived CD8 T cells. To analyze the epitope recognition of spleen-derived CD8 T cells, each of the 15 segments of the TMEV library was co-transfected with the D^b class I molecule in Cos-7 cells. Magnetically sorted CD8 T cells isolated from the spleens of 7 day TMEV-infected C57BL/6 mice were cultured with Cos-7 cells co-transfected with each of the 15 library segments and D^b. Twenty-four hours after the addition of CD8 T cells, a sample of culture media was taken from each well to be analyzed for IFN-γ expression. Despite low numbers of detectable D^b:VP2_{121-130} epitope CD8 T cells isolated from the spleen (Figure 4A), there was significant
response towards the TMEV genomic segment 2 encoding the immunodominant VP2_{121-130} peptide, as confirmed by IFN-γ expression (Figure 4B). These experiments demonstrated that despite the low numbers of Db:VP2_{121-130}–specific spleen-derived CD8 T cells, epitope recognition remains exclusively focused towards this immunodominant epitope. In addition, no other TMEV genome segments encoded additional epitopes detectable among spleen-derived CD8 T cells with our library system (Figure 4B). Using this system, we have determined that the central and peripheral CD8 T cell compartments appear have similar epitope specificity. Clonal expansion and identical T cell receptor use among CD8 T cells in cerebral spinal fluid and peripheral blood has also been reported in human studies (24).
Discussion

In this study we have developed an unbiased molecular approach for determining CD8 T cell epitopes without the use of culture systems. Unlike the use of peptide MHC tetramer technology, this system has the potential to analyze the full inflammatory CD8 T cell response toward multiple epitopes simultaneously. Using this system in TMEV-infected C57BL/6 mice, we have determined that the CD8 T cells isolated from both the brain and the spleen peripheral lymphoid compartment are specific for the same region of TMEV. This is the first direct comparison of spleen and brain CD8 T-cell responses using a library approach to analyze all potential class I epitopes. With the knowledge that peripheral CD8 T cells are specific for the same MHC class I target in the CNS emerges the possibility that a similar approach could be designed for human work using a much more complex, randomly generated library, as has been performed by others (10, 14). Such a system could incorporate HLA-E and the detection of suppressor cytokines in addition to classical HLA molecules and detection of Th1 cytokines. A humanized system would be a marked advantage over current systems that require the culture of CD8 T cells from peripheral blood or CSF cells which are subject to activation-induced cell death and promiscuity of the T cell receptor (10, 26). In addition, our system could also aid in the characterization of “driver clones,” as recently described in the EAE model of MS by Menezes et al. (18). Whether such clones exist in other murine models of MS or in human MS is unknown at this time. A potential pitfall in the proposed future use of this technology in suppressor CD8 T cells is that there may be non–epitope-specific suppressor CD8 T cells, as recently demonstrated (3, 4). In addition, there may be
unknown effects of antigen processing related to the interspecies mismatch between monkey cells (Cos-7) and murine or human cells.

Randomly generated cDNA expression libraries using the Cos-7 system have been used to define epitopes in complex diabetes models as well as large herpes viruses (15, 32). In general, these approaches use random cDNA libraries generated through incomplete Sau3AI 4–base pair cutting enzymatic digestion. The resulting digested cDNA fragments are cloned into pCDNA3.1 expression vectors in three separate reading frames. Pools of these randomly generated clones are then transiently expressed along with a class I molecule of interest. This approach uses a high throughput method to transiently express all potential CD8 T cell epitopes along with a class I of interest. cDNA clones are identified only after demonstration of successful stimulation of CD8 T cells. In this manner, all potential epitopes have the capacity to be presented by a chosen HLA molecule also co-transfected into the Cos-7 cell. The resulting library therefore is interchangeable with other class I molecules. Such a system could incorporate HLA-E and the detection of suppressor cytokines in addition to classical HLA molecules and detection of the Th1 cytokines.

The two prevailing hypotheses that clonally expanded CD8 T cell subsets are acting as suppressors or effectors in MS demonstrates the importance of ultimately defining class I:peptide epitopes. Defining epitopes in CNS inflammatory diseases will be critical to both understand their role in disease as well as manipulate their function to positively impact disease outcome. We also demonstrated that nearly undetectable CD8 T cell populations in the periphery of the mouse are focusing on the same response as the
brain, a novel finding of our study. The observation that peripheral CD8 T cells are focused on the same target epitope as CNS-infiltrating CD8 T cells also demonstrates that a similar more complex organ specific cDNA library approach could potentially be applied to clinical studies in MS.
Materials and methods

Animals: Five week old male C57BL/6 mice were obtained from Jackson laboratories (Bar Harbor, Maine). Mice were anesthetized with isoflurane and were intracranially infected with the Daniels strain of TMEV. Seven days post infection the animals were euthanized, and tissue was harvested according to University of Cincinnati Institutional Animal Care and Use Committee standards.

Generation of the TMEV library: Fifteen overlapping segments were amplified from the pDAFL plasmid containing the DA strain of TMEV using high-fidelity polymerase chain reaction (PCR), as presented in Table 1. Each segment was enzymatically digested then inserted into the pcDNA 3.1 His A vector (Invitrogen, Carlsbad, CA). The pcDNA 3.1 His A was digested with either EcoRI and XbaI to accommodate ligation of segments 1, 3, 5, 6, 7, 9, 10, 11, 12, 13, and 15 or BamHI and XbaI to accommodate ligation of segments 2, 4, and 8. In addition, the genomic sequence encoding the immunodominant TMEV peptide VP2_{121–130} was inserted into the pcDNA 3.1 His B vector.

Efficient co-transfection of Cos-7 cells with class I molecule and green fluorescent protein: Cos-7 cells (American Type Culture Collection number CRL-1651, African green monkey kidney cells) were plated into Costar 96-well culture plates (Corning Inc., Corning, NY) at approximately 70% confluency and incubated overnight. Cells were transfected using Fugene-6 (Roche Indianapolis, IN) according to the manufacturer's protocol. Cos-7 cells were co-transfected with green fluorescent protein (GFP) MigR1 vector and a vector containing either the D^b or K^b MHC class I molecules (provided by Nilabh Shastri, University of California at Berkley, Berkeley, CA) which
were expressed by the C57BL/6 strain of mouse. GFP MigR1 was co-transfected as a visual aid to distinguish wells that demonstrated efficient co-transfection. Peak GFP expression occurred in Cos-7 cells within 24–48 hours after transfection.

**Isolation of brain mononuclear cells:** Brains were pushed through 100-μm cell strainers into RPMI and 700 μg of collagenase type 4 (Worthington Lakewood, NJ) was added to each 5-ml quantity of slurry. Slurries were then incubated in a water bath at 42°C for 45 minutes. Each 5 ml of slurry was then added to Nalgene 50-ml ultra–high-speed centrifuge tubes (Nalge Nunc International, Rochester, NY) containing 1ml of 10X PBS, 9 ml of Percoll, and 35 ml of RPMI. Cell suspensions were then spun at 10,000 rpm (Sorvall SS-34 rotor) for 30 minutes. A lymphocyte layer was present in approximately the bottom 5 ml of media solution. Excess solution is aspirated off the lymphocyte layer. The lymphocyte layer was then resuspended into Falcon conical tubes, (Becton Dickinson, Franklin Lakes, NJ) and RPMI was added until 50 ml of total volume was reached. Cell suspensions were then spun at 1500 rpm for 10 minutes in a Sorvall Legend RT tabletop centrifuge (Thermo Scientific, Waltham, MA). Media was aspirated off and cell pellets were resuspended in RPMI media.

**Isolation of whole spleen cells:** Spleens were pushed though 100-μm cell strainers into RPMI 1640. Cell suspensions were then spun at 1500 rpm for 10 minutes in a Sorvall Legend RT tabletop centrifuge. Media was aspirated off and cell pellets were resuspended in 2ml RPMI and 3 ml ACK (0.15 mol/l NH4Cl, 1.0 mol/l KHCO3, 0.1 mmol/l Na2-ethylenediaminetetraacetate). Cell suspensions were then spun at 1500 rpm for 5 minutes. Media was aspirated off and cell pellets were resuspended in RPMI 1640.
Purification of CD8+ cells: Isolated lymphocytes from the brain and spleen were resuspended in magnetic-activated cell separation (MACS) buffer (1×PBS and 0.5% bovine serum albumin) and incubated with MACS beads anti-CD8+ antibody (Miltenyi Biotec, Auburn, CA) at 4°C for 15 minutes. Cell suspensions were then diluted with 20 times additional MACS buffer and spun at 1500 rpm for 5 minutes on a tabletop centrifuge. Cell pellets were then resuspended in MACS buffer. Cell suspensions were then filtered through MACS LS separation columns (Miltenyi Biotec) according to the manufacturer's protocol.

Intracellular staining of splenocytes: Splenocytes were isolated from 7-day TMEV-infected animals as discussed above. Cells were stimulated with either VP2_{121–130} peptide or E7 control peptide. Cells were treated according to the manufacturer's protocol using the BD Cytofix/Cytoperm Plus Fixation/Permeabilization kit (with BD GolgiPlug protein transport inhibitor containing brefeldin A; BD Bioscience, San Diego, CA). Cells treated with E7 control peptide yielded no cells positive for interferon (IFN)–γ (data not shown).

Staining cells for flow cytometric analysis: Cells were resuspended in fluorescence activated cell sorter (FACS) buffer (1X phosphate-buffered saline solution, 0.025% sodium azide and 1% fetal calf serum). Brain-derived lymphocytes were stained with D^b:VP2_{121–130} tetramer for 40 minutes followed by 20 minutes of staining with anti-CD8 antibody. Cells were then washed twice with FACS buffer and resuspended in 1% paraformaldehyde. Cos-7 cells were stained with biotinalated anti-K^b,D^b primary antibody (BD Pharmingen clone # 28-8-6) for 20 minutes. Cells were washed with FACS buffer and incubated with streptavidin phycoerithrin for 20 minutes. Cells were then
washed twice with FACS buffer and fixed with 1% paraformaldehyde. All cell samples were analyzed on a BD LSR II flow cytometer.

**Enzyme-linked immunoabsorbent assay:** The eBiosciences Mouse IFN-γ Femto-HS enzyme-linked immunoabsorbent assay (ELISA) kit (cat# 88-8314) (eBiosciences, San Diego, CA) was used to detect the presence of IFN-γ in the media of wells containing virus-specific CD8 T cells plated onto Cos-7 cells expressing GFP and an MHC class I/TMEV peptide complex. IFN-γ detection was performed according to the manufacturer's protocol.

**Statistical analysis:** Data were analyzed with the statistical package in Microsoft Excel using Student’s t-test. The level of significance was set at ≤0.05.
References


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Acknowledgments

We acknowledge Nilabh Shastri for providing the K\textsuperscript{b} - and D\textsuperscript{b}-expressing plasmids, and likewise thank Feng Gao for technical assistance. This work was supported by National Institutes of Health grant RO1 NS058698, a National MS Society Pilot Grant, Waddell Center for MS, and intramural funds. The authors have no conflicting financial interests.
Table 1- Overlapping segments amplified from pDAFL plasmid containing Daniels strain of Theiler’s murine encephalomyelitis virus (TMEV). Fifteen overlapping segments were amplified from the pDAFL plasmid containing the TMEV genome using high-fidelity polymerase chain reaction. Segments were then digested with the specified restriction enzyme as defined by primer sequences and inserted into the pcDNA 3.1 His A vector to accommodate transfection into the Cos-7 cell line. The TMEV sequences overlap by 30 base pairs to ensure that all potential CD8+ T-cell epitopes are processed for antigen presentation. Note that the L* segment encodes a protein from an alternative reading frame important to TMEV infection.
Figure 1- Molecular approach to detect CD8+ T-cell epitope specificity directly *ex vivo*. Overlapping segments were amplified from the Theiler’s murine encephalomyelitis virus (TMEV) genome as noted in (A) and Table 1. In (B), overlapping TMEV genomic segments were cloned into pcDNA 3.1 His A. Individual wells of Cos-7 cells are co-transfected with either major histocompatibility (MHC) class I molecule K\(^b\) or D\(^b\) and a specific TMEV genomic segment. At 24–48 hours after transfection, Cos-7 cells express TMEV peptidepresenting MHC class I molecules. Upon epitope recognition, central nervous system (CNS)–infiltrating and spleen-derived CD8+ T cells express detectible levels of interferon (IFN)–γ. This molecular approach is designed to enable recognition of all TMEV restricted CD8+ T-cell responses.
Figure 2- Flow cytometry analysis of efficient class I and green fluorescent protein (GFP) co-transfection in Cos-7 cells. Co-transfected Cos-7 cells were stained with antibody that binds both the D<sup>b</sup> and K<sup>b</sup> class I molecule. Shown are a representative well of (A) cells transfected with empty pcDNA 3.1 His A vector, (B) D<sup>b</sup> plus GFP expression vectors, and (C) K<sup>b</sup> plus GFP expression vectors. Note that cells within the upper right quadrant denotes successful co-expression of GFP and class I molecules.
Figure 3- Central nervous system (CNS)–infiltrating CD8+ T cells recognize the immunodominant D^b:VP2\textsubscript{121–130} epitope as determined by our Cos-7 co-transfection system. (A) Enzyme-linked immunoabsorbent assay was used to detect interferon (IFN)–γ expression by central nervous system (CNS)–infiltrating CD8+ T cells co-cultured with Cos-7 cells co-transfected with D^b plus pcDNA 3.1 His A/VP2. D^b plus empty pcDNA 3.1 His A vector resulted in no IFN-γ expression. In (B), D^b:VP2\textsubscript{121–130} tetramer staining demonstrated that at 7 days postinfection, approximately 55% of all brain-infiltrating CD8+ T cells were specific for the VP2\textsubscript{121–130} epitope confirming the results in (A). These data demonstrate that VP2\textsubscript{121–130} peptide was successfully expressed, processed, and presented by D^b class I molecule demonstrating the feasability of our co-transfection molecular approach to stimulate the response of ex vivo isolated CD8+ T cells. Values presented in (A) are the mean absorbance [a] of two wells from a single preparation. Bars are mean ± s.e.m.; *P<0.001
Figure 4- Spleen derived CD8 T cells are restricted to the epitope derived from the same Theiler’s-murine encephalomyelitis virus (TMEV) genomic segment as central nervous system (CNS)-infiltrating CD8 T cells. (A) Lymphocytes isolated from the spleen of 7-day TMEV-infected mice were cultured for 4 hours with VP$_{121-130}$ peptide and assessed for interferon (IFN)–γ expression with intracellular cytokine staining. No IFN-γ–positive cells were found in wells stimulated with the E7 control peptide (data not shown). In (B), CD8 T cells isolated from the spleen were co-cultured with Cos-7 cells co-transfected with D$^b$ and all 15 individual TMEV genomic segments. CD8 T cells demonstrated specificity for segment 2 of the TMEV genome, which encodes the immunodominant VP$_{121-130}$ peptide, when compared with stimulation with Cos-7 cells co-transfected with D$^b$ and empty pcDNA 3.1 His A vector as measured by the absorbance [a] from enzyme-linked immunoabsorbant assay IFN-γ detection (*P < 0.0004). All wells in (B) were performed in quadruplicate from a single preparation.
Chapter three

**Rapid formation of extended processes and engagement of neurons by CNS infiltrating CD8 T cells**

**portions of this work adapted from:**

McDole, J., Danzer, S., Pun, R. Y. K., Chen, Y., Pirko, I., Johnson A. J. “Rapid formation of extended processes and engagement of neurons by CNS infiltrating CD8 T cells” (manuscript in preparation)
Abstract

A fundamental question in neuroimmunology is the extent CD8 T cells actively engage neurons. In the Theiler’s murine encephalomyelitis virus (TMEV) model of multiple sclerosis, an effective CNS-infiltrating antiviral CD8 T cell response protects from demyelinating disease. However, the specific CNS cell types engaged by these protective CD8 T cells in TMEV-resistant strains remains unknown. We employed 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:VP2_{121-130}. 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 \mu m in length. Using live tissue imaging we determined these T cell extended processes (TCEPs) could be rapidly formed and were associated with migratory behavior through CNS tissue. 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 by these brain infiltrating lymphocytes using live tissue imaging.
**Introduction**

The direct engagement of neurons by CD8 T cells is a putative mechanism to both clear neurotropic virus infections 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 detectible levels of MHC class I protein despite high levels of mRNA expression (8, 12, 18, 30, 32). Other studies have concluded that MHC class I molecules can be up regulated in neuronal cultures (25, 43). 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 (13). The conclusion from the above studies implies that underlying class I expression is necessary for full physiologically functioning neurons. However, expression 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 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 (16, 35). 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(16, 35). In addition to the genetic 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, VP2_{121-130}, presented in the context of the D^b class I molecule (6, 15, 28). Despite the importance of this interaction in protecting against demyelination, the specific 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 by us and others (17-19).

CD8 T cell engagement of target cell types is also dependent on motility through CNS tissue. Lymphocyte motility in live tissue has been characterized within lymphoid organs and among spinal cord-infiltrating myelin basic protein-specific CD4 T cells (7, 20). These studies determined that alterations in lymphocyte morphology accompany cellular movement (29). While clearly documented in lymph nodes, the importance of morphological phenotypes and motility among CD8 T cells during the effector phase of virus-infected brain remains undefined. Here, we address the importance of antigen specific CD8 T cell morphology and its relationship to motility using our well characterized TMEV model system.

Advances in confocal microscopy have enabled studies of CD8 T cells engagement with virus-infected CNS cell types (2, 24). In this study, we utilize a novel 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:VP2_{121-130} epitope-specific CD8 T cells engage specific TMEV-infected CNS cell types. We also utilize this highly efficient transfer strategy to visualize CD8 T cell morphology and motility in the CNS tissue using live tissue imaging.
Results

Adoptively transferred brain-infiltrating CD8 T cells are predominantly virus specific

CD8 T cell specificity for the immunodominant D\textsuperscript{b}:VP\textsubscript{2121-130} TMEV epitope in the C57BL/6 mouse strain during acute TMEV infection has been well characterized (6, 14, 15, 17, 28, 39). To determine the target of these antiviral CD8 T cells in the CNS during acute TMEV infection, we developed an adoptive transfer method, paired with confocal microscopy (Figure 5A). Through this analysis, we determined that adoptively transferred GFP+ CD8+ cells became highly specific for the immunodominant D\textsuperscript{b}:VP\textsubscript{2121-130} epitope. Ninety-two percent of GFP expressing cells recovered from the CNS also expressed CD8 protein (Figure 5B). Of this GFP+ CD8+ population, approximately 93% of CD8+ cells were specific for the D\textsuperscript{b}:VP\textsubscript{2121-130} epitope (Figure 5B). This high level of epitope dominance was not the result of non-specific binding to tetramer, as GFP+ CD8+ cells did not appreciably stain with mock control D\textsuperscript{b}:E7 tetramer (Figure 5C) consistent with previous reports (10, 26). This high percentage of adoptively transferred CD8+ cells that became specific for the D\textsuperscript{b}:VP\textsubscript{2121-130} epitope provided us with high confidence that GFP expression would serve as a suitable marker for detection of virus specific CD8 T cells in the CNS using confocal microscopic analysis of the adjacent hemisphere.

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Translation of TMEV proteins is predominately in neurons during acute infections

We determined through flow cytometric analysis of the left hemisphere that 93% of adoptively transferred GFP+ CD8 T cells were antiviral (Figure 5B). 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 tissue slices obtained from the opposing right hemisphere. Using confocal microscopy, we observed TMEV protein translation in the hippocampus, striatum, hypothalamus and cortex. We counted 150 randomly distributed TMEV positive cell bodies from 10 tissue slices through the hippocampus taken from 4 animals; 142 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 6A-C). In addition, 150 randomly distributed GFAP positive cells from 10 tissue slices through the hippocampus from 4 animals were analyzed for TMEV staining. Among these cells, none stained positive for TMEV protein, indicating activated astrocytes were not actively translating virus protein (Figure 6D-F). Furthermore, TMEV infected neurons often presented with dendritic beading indicative of cellular degeneration (Figure 6G-I). This analysis demonstrated that acute TMEV infection in C57BL/6 mice is predominately localized to neurons.

CD8 T cells form immune synapses with virally infected neurons

Adoptively transferred CNS infiltrating GFP+ CD8 T cells were observed in close proximity to TMEV infected neurons and their processes (Figure 7A). 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 towards virus infected neurons when in close proximity (Figure 7B-E). Instead, CD8 T cells polarized both CD8 protein and T cell receptor towards neurons in brain regions with known TMEV infection, an observation previously defined to be indicative of immune synapse formation (Figure 7F-M) (2). CD8 T cells in close proximity to TMEV infected neurons also polarized both CD8 protein and granzyme B (Figure 7N-Q).

Despite T cell receptor and granzyme B polarization among proximal CD8 T cells, MHC class I H-2D\(^b\) protein was not highly expressed on neurons (Figure 8). By comparison, class I staining was readily detectable on CD8+ cells (Figure 8A-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 9A-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 10A-D and I-L) and GFP+ cells that formed long projections of varying thickness which we have termed T cell extended processes (TCEPs) (Figure 10E-H and M-P). In Figure 10E, the GFP+ cell shown presents with a TCEP that extends to nearly 45 \(\mu\)m in length. Both round and TCEP presenting GFP+ cells expressed CD3 (Figure 10B and F) and CD8 (Figure 10J and N) protein, confirming their identity as T lymphocytes as predicted by the flow cytometric analysis on the opposite brain hemisphere (Figure 5B). Additionally, both
round and TCEP presenting GFP+ cells presented with an activated phenotype as indicated by granzyme B expression (Figure 10Q-X).

**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 11A 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 hippocampal brain region using live tissue imaging. High endogenous GFP expression among adoptively transferred CD8+ cells enabled clear visualization using real time confocal analysis in live hippocampal slices (Figure 5B). Through this analysis, the potential importance of the different CD8 T cell morphologies became apparent. Round GFP+ cells (previously defined in Figure 10A-D and I-L above) were stationary and maintained a spherical shape for over one hour (Figure 11C, Video 1). GFP+ cells that presented with large segmented TCEPs (previously defined in Figure 10U-X above) were motile and traveled through tissue at a rate of approximately 1-2 μm/min. The specific cell shown in Figure 11D (Video 2) used a “stop-and-go burst pattern” of movement utilizing TCEP formation to accomplish motion. 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 11A and B (Video 3). 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 11E).
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 co-localized with and are tightly apposed to target neurons. Tightly apposed CD8 T cells were observed polarizing granzyme B, TCRβ and CD8 protein consistent with activation and the formation of immune synapses (2, 3). 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 (22). In order to determine the mechanism by which CD8 T cells control TMEV infection and prevent demyelinating disease, we examined the interaction between adoptively transferred GFP+ CD8+ lymphocytes and virus-infected neurons. In vivo polarization 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 (2, 33). In contrast, we determined that cellular engagement with neurons among CD8+ T cells was characterized by the polarization of CD8 protein, granzyme B and TCRβ. These observations are consistent with the previous analysis of CD8 T cell engagement with adenovirus infected astrocytes (2). Whether utilization of granzyme B by CD8 T cells contributes to the degenerative change observed in target neurons is currently under investigation, as TMEV infection alone can result in neuronal death (40). These studies
also indicate that CD8 T cells have the capacity to simultaneously engage multiple targets as has been reported previously (24).

Consistent with previous reports, we did not observe high levels of MHC class I protein on the surface of neurons (17, 18, 21). However, these studies determined that T cell receptor could polarize towards neurons regardless of undetected 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 immunostaining. It is well documented that cellular targets of T cells with greatly reduced MHC molecule expression retain the capacity to form synapses (9, 11). 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 since these CD8 T 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 results in the preservation of axons and motor ability in chronically demyelinating, TMEV-infected animals (17, 34, 41). Recently, we determined that CD8 and perforin deficient mice had diminished capacity to develop T1 hypointensities in TMEV infection as visible by MRI (31). Additional MR spectroscopy analysis on the brains of these mice revealed that these T1 hypointensities are likely the result of axonal loss (31). Furthermore, analysis of human MS lesions has correlated the presence of CD8 T cell infiltration with axonal damage (4). Other human diseases such as Rasmussen encephalitis and progressive multifocal leukoencephalopathy also link CD8 T cells to neuronal pathology (27, 37). Data presented in the present 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 5 times in length as compared to width were reported (29). 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 5 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 motilities of both naïve and antigen-challenged T lymphocytes within lymphoid compartments are now being studied. Specifically, the motility of myelin basic protein-specific CD4 T lymphocytes in the mouse spinal cord has been analyzed (20). Kawakami et al. determined that
autoimmune CD4 T cells undergo various morphological changes and move at speeds up to 25 μm/min. “Resting” lymphocytes within lymphoid compartments move at an average speed of 10-12 μm/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, utilizing a “stop-and-go burst pattern” of movement (29). However, virus-specific CD8 T cells analyzed in these studies moved at greatly reduced speeds as compared to previous studies of lymphocytes imaged in the lymph node. The maximum speed observed among our brain-infiltrating GFP+ cells was approximately 1-2 μm/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. Alternatively, reduced speeds may be the result of temperatures cooler than those found within the lymph organ.

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 (38). 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 following antigenic challenge (20).

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 (5, 23). The engagement of CD8 T cells with neurons may therefore protect against demyelination syndrome but may also perpetuate cell death found in neurological diseases characterized by infiltration of these lymphocytes, including MS.
Materials and Methods

Mice: C57BL/6-Tg(UBC-GFP)30Scha/J (stock number 004353) females were bred in-house in the University of Cincinnati Laboratory Animal Medical Services (LAMS) facility. 5 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 100µm filter. CD8+ cells were purified from the resulting lymphocyte population using MACS LS cell purification columns (Miltenyi Biotec, Auburn, CA) according to the manufacture’s protocol resulting in 95% purity as determine by flow cytometric analysis (data not shown). C57BL/6J mice were irradiated with 400 rads of γ-radiation then received 10^6 CD8+ enriched cells via tail-vein injection.

Virus infection: One day after adoptive transfer, mice were anesthetized with isoflurane and intracranially infected with the 2 X 10^6 PFU of Daniel’s strain TMEV (36). 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: $D^b$:VP$_{2121-130}$ and $D^b$:E7 tetramers were made as previously described (1, 10, 17). H-2Db and human β2m 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 VP$_{2121-130}$ 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-steptavidin (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 2.

Isolation of brain lymphocytes: Brains were strained through a nylon mesh 100μm filter into RPMI (MediaTech Inc., Herndon, VA) and 700 ug 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 1ml of 10X PBS, 9 ml of Percoll (Sigma-Aldrich GmbH, Steinheim, Germany), and 35 ml of RPMI. Cell suspensions were then centrifuged at
10,000 rpm (Sorvall SS-34 rotor) for 30 minutes. 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 suspensions were then centrifuged at 1500 rpm 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). 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. Because TCRβ and MHC class I antibodies had low efficacy in fixed tissue, fresh frozen tissue was employed. 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°C 95% ethanol for 15 minutes. Slides were incubated with primary antibodies overnight followed by 4 X 5 minute rinses. Slides were then incubated with secondary antibodies for 1 hour followed by 4 X 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 (LeicaMicrosystems CMS GmbH, Mannheim, Germany) was used to analyze images. Three-dimensional Z-series stacks were captured at 0.25μm.

Time-lapse confocal imaging: Briefly, brain slices were prepared for time-lapse confocal imaging by anesthetizing animals with ketamine (100mg/kg) followed by decapitation. Brains were removed, immersed in ice-cold artificial cerebral spinal fluid (ACSF) (124 mM NaCl, 3 mM KCl, 2 mM MgSO₄, 2 mM CaCl₂, 1.5 mM NaH₂PO₄, 26 mM NaHCO₃, 10 mM 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 μm 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 ludin chamber warmed to 36°C to maintain temperature stability. Slices were screened under epifluorescent illumination and 10X (NA 0.3) magnification to locate GFP expressing lymphocytes. Magnification was then increased to 40X (NA 0.6) or 63X (NA 1.3) to focus on specific cell(s) of interest.
References


Theiler's virus recognize H2Db molecules complexed with a viral VP2 peptide lacking a consensus anchor residue. J. Virol. 71:5244-5250.


Acknowledgements

A special thanks to Markus Simon of the Max-Planck Institute of Immunobiology Freiburg, Germany for providing granzyme B antibody and Moses Rodriguez of the Mayo Clinic Rochester, Minnesota for providing TMEV antibody. This work was supported by NIH grant R01 NS058698 and by intramural funds at the Waddell Center for MS at the University of Cincinnati.
Figure 5- Adoptively transferred GFP+ CD8+ splenocytes infiltrate the CNS and become highly focused towards the immunodominant D<sup>b</sup>:VP2<sub>121-130</sub> virus epitope during acute TMEV infection. (A) Schematic for adoptive transfer system to analyze CNS infiltrating CD8 T cells using immunohistochemistry and flow cytometry. 10<sup>6</sup> magnetically purified spleen-derived CD8+ cells isolated from the GFP-expressing C57BL/6 mice were intravenously administered to partially irradiated wildtype C57BL/6 mice. One day later, mice were intracranially infected with TMEV. At seven days post-infection, 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. Adoptively transferred GFP+ cells were analyzed for CD8 expression and (B) D<sup>b</sup>:VP2<sub>121-130</sub> tetramer or (C) negative control D<sup>b</sup>:E7 tetramer staining (n = 4; 2 experiments).
Figure 6- 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 (A) TMEV and (B) NeuN demonstrated a high level of overlap in (C) merged images (n = 4; 3 experiments). Labeling for (D) TMEV never overlapped with (E) GFAP labeling as shown by (F) merged images (n = 4; 3 experiments). Neurons which stain positive for TMEV protein presented with the onset of (G) dendritic beading while others were (H) heavily degenerated and (I) fragmented (n = 10; 7 experiments). Bars: 25 μm.
Figure 7- 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 (n = 16; 8 experiments). Shown in (B) is a round GFP+ lymphocyte without (C) CD3 polarization against (D) a TMEV infected neuronal body. These images are merged in (E) (n = 5; 4 experiments). Both (F and J) CD8 protein and (G and K) T cell receptor polarized towards (H and L) neurons highly indicative of an immune synapse (n = 4; 2 experiments). Merged images are shown (I and M). Enhanced (N) CD8 expression and (O) granzyme B polarization was present at the point of tight apposition between 2 CD8 T cells and (P) a TMEV infected neuron and the (Q) merged images (n = 8; 4 experiments). Bars: (A) 25μm; (B-Q) 10μm.
Figure 8- Neurons have reduced surface H-2D\textsuperscript{b} class I molecules as compared to lymphocytes. (A and E) NeuN positive neurons did not express detectible levels of H-2D\textsuperscript{b} class I molecule. By comparison, (B and F) CD8\textsuperscript{+} cells directly adjacent to neurons had high levels of (C and G) class I molecule expression and the merged images are shown in (D and H) (n = 4; 2 experiments). Bars: 10μm.
Figure 9- Quantification of CD8 protein polarization towards 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 towards two separate neurons simultaneously. Note that arrows A, B, and C in microscopy images correspond with peaks of luminescent intensity [I] (n = 8; 4 experiments). Bars: 10 μm.
Figure 10- Adoptively transferred CD8 T cells are morphologically diverse. Distinct morphological phenotypes were found among adoptively transferred cells. (A and I) Round and (E and M) TCEP presenting GFP+ cells express (B and F) CD3 or (J and N) CD8 protein. Neurons are distinguished by (C and G) TMEV or (K and O) βIII tubulin staining. The merged images are shown in (D, H, L and P) (n = 8; 4 experiments). Both (Q) round and (U) TCEP presenting CD8 T cells were activated as indicated by (R and V) granzyme B expression. GFAP staining is shown in (S and W) as a reference. Merged images are shown in (T and X) (n = 8; 4 experiments). Bars: 10μm.
Figure 11 - Morphology and TCEP formation is consistent with movement as demonstrated by live tissue imaging. Adoptively transferred (A) GFP+ cells expressed (B) CD18, a component of the LFA-1 adhesion molecule complex, on the TCEP. 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. In (C), cells with round morphologies did not demonstrate movement or formation of projections for a period of 60 minutes. In (D), GFP+ cells that were actively moving presented with a segmented morphology and traveled through tissue at a rate of approximately 1-2 μm/min. GFP+ cells were capable of (E) rapid formation of TCEPs within 2 minutes that extend two cell lengths. This cell rapidly extended and contracted these projections while remaining largely stationary over the time course observed (n = 2; 2 experiments). (Asterisks denote a relative point of origin in the tissue slice.) Bars: (C) 25μm; (D and E) 10μm.
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**Table 2- Antibodies used for immunohistochemistry stains in this study.** Antibodies are listed by name and include relevant manufacturing information. All primary antibodies utilized throughout this study were diluted in a solution of 5% normal goat serum and 0.5% Igepal in 1X PBS at a 1:500 concentration. Secondary antibodies were diluted in the same solution and used at a 1:750 concentration.
Video 1- Round, adoptively transferred GFP+ cells remain immobile for extended periods of time. Morphological changes and movement of adoptively transferred GFP+ cells is negligible for over 2 hours. The left GFP+ cell changes shape and moves out of the field of view after approximately an hour. Each movie frame represents a 50 μm z-stack consisting of 5 progressive scans, 10 μm apart using time-lapse confocal microscopy. Frames were captured every 20 minutes. Movie is shown at 3 frames/sec (n = 2; 2 experiments) (green = GFP).
Video 2- TCEP presenting, adoptively transferred GFP+ cells demonstrate movement. The GFP+ cell in the video undergoes morphological changes during the stop-and-go burst pattern of movement. TCEP formation preceded movement. Similar morphologies were observed in fixed tissue among CD8+ cells. Each movie frame represents a 50 μm z-stack consisting of 5 progressive scans, 10 μm apart. Frames were captured every 2 minutes. Movie is shown at 3 frames/sec (n = 2; 2 experiments) (green = GFP).
Video 3- Adoptively transferred GFP+ cells are capable of rapid morphological changes. This video demonstrates rapid extension and retraction of TCEPs on a single adoptively transferred GFP+ cell. Each movie frame represents a 50 μm z-stack consisting of 5 progressive scans, 10 μm apart. Frames were captured every 2 minutes. Movie is shown at 3 frames/sec (n = 2; 2 experiments) (green = GFP).
Chapter four

General Discussion: CD8 T cells engage neurons during acute TMEV infection**

**portions of this work adapted with permission from:


McDole, J., Danzer, S., Pun, R. Y. K., Chen, Y., Pirko, I., Johnson A. J. “Rapid formation of extended processes and engagement of neurons by CNS infiltrating CD8 T cells” (manuscript in preparation)
Neuronal damage is considered to be a primary feature of many conditions including paraneoplastic disorders, Rasmussen’s encephalitis and MS. Neuronal injury and loss have far-reaching cognitive and behavioral consequences in patients making alleviation of this form of pathology a primary therapeutic goal. CD8 T cells have been implicated to contribute to neuronal damage in these disorders due to their proximity to neurons in tissue (4, 34, 38). Discovering the CD8 T cell epitopes in these disorders has potentially widespread therapeutic applications for reducing this neuronal injury as has been demonstrated in the TMEV animal model of MS (17). Here, we have presented a molecular approach for determining CD8 T cell epitopes that can potentially be translated to human conditions including MS. Further, the potential mechanism utilized by CD8 T cells to render neuronal injury has remained elusive. Understanding this mechanism would provide major insight into the development of treatments that could inhibit the destructive effects that CD8 T cells potentially have in these conditions. In these sets of studies we provide strong evidence to support the hypothesis that CD8 T cells can directly engage neurons. Our data demonstrate for the first time the polarization of TCRβ against target neurons, supporting the hypothesis that CD8 T cells form traditional immune synapses with this CNS cell type. Additionally, we show CD8 T cells polarizing granzyme B against target neurons. This suggests that this type of engagement elicits an effector function from the CD8 T cell. This work is significant because it offers insight into the potential mechanism by which CD8 T cells could perpetuate neuronal damage in a variety of disease conditions.

The CD8 T cell epitopes in MS remain undefined (25, 26). Discovery of these epitopes could contribute to potential therapies as has been previously explored by our
lab in the TMEV model of MS (17). In these earlier studies we showed that
administration of the virus peptide VP2_{121-130} before TMEV infection inhibited the
expansion of CD8 T cells specific for this epitope. Inhibition of this CD8 T cell
population in chronically demyelinated animals resulted in sparing of motor function
compared to animals with an intact population of expanded CD8 T cells specific for
VP2_{121-130} (17). In the studies presented here we have provided a molecular approach for
determining epitopes among CD8 T cells in our TMEV model (26). This approach is
unbiased because it does not require the culturing of CD8 T cells which can skew epitope
specificity. Instead, epitope-specific CD8 T cells are directly extracted from the animals
and plated onto Cos-7 cells. These Cos-7 cells express MHC class I molecules and
TMEV protein from overlapping segments of the virus genome library. Presentation of
the appropriate virus peptide/MHC class I epitope complex by Cos-7 cells provides a
target that can then be engaged by CD8 T cells. IFN-γ expression, a result of this
engagement, can then be detected by ELISA. For studies presented here, baseline IFN-γ
expression was determined by the CD8 T cell response to Cos-7 cells transfected with
class I and an empty viral cassette containing no segments from the TMEV genome
library.

In our studies we extracted a population of CNS-infiltrating CD8 T cells from 7-
day TMEV-infected C57BL/6 mice previously established to be specific for the TMEV
epitope VP2_{121-130}. Using our molecular approach we confirmed the specificity of this
CD8 T cell population in a proof-of-principle study, demonstrating the effectiveness of
our paradigm. Using this same approach in 7-day TMEV-infected animals we
demonstrated that a population of spleen-derived CD8 T cells also demonstrated
specificity for the VP2121-130 epitope. This suggests that epitope specificity among peripheral CD8 T cells is similar to that of CNS-infiltrating CD8 T cells which potentially perpetuate pathology and clinical dysfunction. CD8 T cells traffic from the spleen into the blood, making the blood an excellent source for epitope-specific CD8 T cells.

These findings also appear to be true in humans. While their epitope specificity was unknown, studies utilizing single-cell PCR and complementary determining region 3 spectratyping demonstrated that clonally expanded CD8 T cells were present in the blood, CSF and CNS of MS patients (1, 35, 36). These finding have fundamental relevance for translating our molecular approach of epitope discovery into MS patients. Utilizing our system, CD8 T cells could be non-invasively isolated from the blood of MS patients, immortalized to preserve TCR specificity and then screened against large libraries of potential epitopes. Once CD8 T epitopes are found in patients, these peptides could be therapeutically administered. In theory, these peptides could bind to the TCR of pathogenic CD8 T cells and without accompanying stimulatory molecules from an APC, potentially induce anergy and cell death as has been demonstrated in mouse models (17).

A project designed to identify the CD8 T cell epitope specificities in MS patients would be extensive and is not without challenges. The screening process would be immense not only because of the large protein library that would have to be generated but also because human MHC class I molecules are highly variable (36). This results in a high number of potential combinations of class I and peptide. This is a notable pitfall. Nevertheless, it has been demonstrated that antigens can be defined using an organ-specific DNA library in a mouse model of type I diabetes (43). A similar approach utilizing MS lesions could
therefore be possible. Such a library would be inclusive of both pathogenic and synegenic antigens. While such an approach is daunting, currently it appears that the approach put forward in this thesis would have a marked advantage in determining the CD8 T cell epitope specificity in humans because it would reduce the bias of culturing T cells with the target antigens commonly studied.

Motility is critical to a lymphocyte’s ability to interact with other cells. While lymphocytic motility had been clearly explored in the lymph nodes, little is known about the movement of brain-infiltrating CD8 T cells under viral infection (29). Acute TMEV infection in C57BL/6 mice provided an excellent model to explore this topic of research. To better assess CD8 T cell movement we developed an adoptive transfer technique which allowed the observation of virus-specific CD8 T cells via live tissue imaging without the need of a TCR transgenetic mouse. To accomplish this we utilized a mouse on a C57BL/6 background that expresses GFP under the ubiquitin promoter. This results in a mouse that expresses GFP in all nucleated cells. To our advantage, T cells express particularly high levels of GFP in this animal making them easily detected by fluorescent imaging. In our adoptive transfer technique wild type C57BL/6 mice are irradiated in order to reduce the number of adaptive immune cells within the animal. This effectively creates “immunological space” which allows for the successful transfer of GFP+ CD8+ cells isolated from the spleens of the GFP-expressing mice discussed above. Once transferred, these GFP+ cells were able to function as a part of the wild type animal’s immune system.

We observed that 7 days after TMEV infection these adoptively transferred GFP+ cells trafficked successfully into the brain of wild type C57BL/6 animals. This
observation was made by both FACS and immunostaining. FACS revealed that approximately 93% of GFP+ CD8+ cells were specific for TMEV. This high level of viral specificity is an advantage to this system because we could be determine with a high confidence that GFP+ cells observed in the brain were virus-specific. This allowed us to observe a relatively natural CD8 T cell response in our studies without the need of a TCR transgenic animal. Immunostaining brain tissue slices immediately revealed that most GFP+ CD8 T cells did not resemble the round lymphocytes found in cellular cultures. Rather, they often had an elongated morphology that we deemed T cell extended processes or TCEPs. TCEP-presenting CD8 T cells often expressed high levels of CD18, a component of the adhesion molecule LFA-1, at the tip of the processes. Further, these TCEP-presenting cells were often activated as demonstrated by granzyme B loading. The observation of CD18 expression pattern and granzyme B loading among T cells provided evidence that morphology was likely associated with motility. Indeed, TCEP-like morphologies had been described among actively moving lymphocytes within lymph nodes (10, 29). This prompted us to examine the movement of these cells using time-lapse confocal imaging.

Using this live tissue imaging technique we were able to capture various motility patterns among GFP+ cells. One phenotype observed was round, immotile cells which did not move for periods of time as long as 2 hours. According to previous observations these round, immobile cells were potentially interacting with a target cell (19, 37). Many examples of TCEP-presenting cell types were also observed. One morphological phenotype that we observed actively moved through tissue in a “stop-and-go burst pattern” of movement. This type of movement had previously been observed among T
cells within lymph nodes (29). This provided strong evidence to support the hypothesis that TCEP morphology was associated with movement through tissue. An additional TCEP phenotype observed was that of cells rapidly extending and contracting processes. This phenotype resembled sampling of the cell’s microenvironment. In spite of dynamic morphological changes, these cells remained largely immobile. TCEP morphologies are not associated with T cell movement in culture (10). This may suggest that the specific mechanisms responsible for producing TCEP morphologies are necessary for movement through the dense tissue found in organs, including the brain. Motility phenotypes in the CNS were comparable to those observed within lymph nodes indicating that in spite of the location, these cells share a similar mechanism of movement (19, 37). An intriguing result in our motility studies was that of the speed of the lymphocytes in the brain. In general we found that these cells moved at approximately 1-2 μm per minute while lymphocytes within the lymph nodes were found to move at speeds up to 25 μm per minute. In the lymph nodes studied, it was found that antigen presentation within the lymph nodes tended to slow cells to approximately 11 μm per minute which is still much faster than the lymphocytes we observed in the brain (29). The much slower speeds observed in our studies may be because CNS tissue is more difficult for a lymphocyte to navigate through than the tissue that comprises the lymph node. Alternatively, antigen presentation may be much different in the CNS compared to the lymph nodes which may ultimately result in reduced lymphocytic speeds. This topic remains an exciting avenue of research in our lab.

Our lab and others have demonstrated that neuronal injury and loss occur in the TMEV infection model of MS (15, 40, 41). It was also demonstrated that neuronal injury
is a critical component of motor dysfunction in this model (14, 15, 42). CD8 T cells have been implicated in this kind of neuronal damage (14, 17). However, the mechanism by which CD8 T cells could perpetuate this kind of pathology remains a critical topic of research. In order to address how CD8 T cells could potentially produce neuronal injury and loss we utilized acute TMEV infection in C57BL/6 mice. While this strain of mouse is resistant to long-term TMEV-induced disease, extensive neuronal destruction was observed within 7 days of infection. A very strong and well documented virus-specific CD8 T cell response occurs in the CNS of these mice 7 days post-infection making this an ideal model to study the interaction between CD8 T cells and neurons (6, 16, 17).

Approximately 70% or more of CNS-infiltrating CD8 T cells become specific for the immunodominant TMEV viral peptide epitope VP2_{121-130}. This strong and highly virus-specific response created a strong probability that CD8 T cells would undergo a high rate of interaction with virus-infected CNS cell types under acute TMEV infection.

We first sought to determine which CNS cell types expressed TMEV virus protein. Traditionally, CD8 T cells are the primary cell type which clears viral infections through engaging and killing virally infected cells. Therefore, to establish which CNS cell types could be targeted by CD8 T cells, we needed to determine which CNS cell types expressed TMEV protein. To accomplish this, we performed immunohistochemistry on brain tissue slices of animals infected for 7 days with TMEV. These tissue slices were taken through areas of the hippocampus, a known area of TMEV infection. Previous literature had shown that astrocytes were a primary cell type of infection (24, 39). However, these studies were performed on various strains of mice many weeks after TMEV infection. These studies also tended to focus on susceptible
strains of mice so it was unclear what CNS cell types would be infected with virus. This work also did not employ high resolution confocal microscopy but rather relied on alkaline phosphatase reactions. Such a technique would be affected by inflammation in general, potentially providing false positives. But because these early studies suggest that astrocytes were the most likely candidate for infection, we stained for using GFAP and simultaneously with TMEV specific antibody. We did not observe any overlap between these markers indicating that activated astrocytes did not express viral protein under acute TMEV infection. However, the staining of TMEV produced patterns closely resembled the morphology of neurons. In order to confirm neurons were virus-infected, we immunostained for NeuN, a specific marker of neurons, and TMEV. We found these markers overlapped in 94.7% of cells scored. This strongly indicated that neurons were the primary cell type that expressed TMEV protein, also making them the potential primary target of CD8 T cells.

It may be that TMEV infection starts in neurons in many different strains of mice. In these studies, we used the C57BL/6 mouse strain which is resistant to chronic TMEV-induced disease. These mice clear virus infection over the course of one month (32). Therefore, virus infection may be contained and ultimately cleared from neurons before having the chance to spread to other cell types. Susceptible strains of mice do not clear virus (32). It is therefore possible that other CNS cell types could become infected over time. Now that we have established a high resolution confocal microscopy technique in the well characterized C57BL/6 mouse, future studies in the susceptible strains are now possible leading to insight into the role of CD8 T cells in chronic TMEV-induced disease.
We next sought to establish if CD8 T cells could engage target neurons. Again, we immunohistochemically stained brain tissue taken throughout the hippocampus of 7-day TMEV-infected C57BL/6 mice. Our results revealed that CD8+ lymphocytes were present in areas of viral infection. In order to elucidate if CD8 T cells were directly engaging neurons CD8 protein, granzyme B and TMEV were immunostained. We found that T cells polarized both CD8 protein and granzyme B against TMEV infected neurons. CD8 protein polarization would be expected since it serves as a major accessory molecule for TCR signaling (23). Because of its association with the TCR, CD8 polarization provided evidence that engagement was taking place. Furthermore, polarization of the granzyme B effector molecule provided more evidence to support the hypothesis that CD8 T cells were directly engaging neurons. Granzyme B has the capacity to induce the caspase cascade that results in apoptosis and is only produced when T cells are activated (22). Therefore, polarization of this molecule against TMEV infected neurons is highly indicative of CD8 T cell engagement and elicitation of an effector function. Finally, while these labeling experiments provided evidence of engagement they did not define what kind of immune synapse may be forming between the CD8 T cell and neuron. A traditional immune synapse is dependent on the polarization of TCR (23). The recognition of the MHC class I/peptide complex by the TCR signals the formation of synapse. However, new evidence suggests that TCR independent synapses also form. These non-traditional synapses are not well defined but at least some appear to be dependent on the polarization of IFN-γ (3). Our studies demonstrated elements of both traditional and non-traditional immune synapses. Immunohistochemistry stains revealed that CD8 T cells polarized TCRβ against cells
positive for the neuron-specific marker NeuN. This demonstrated a traditional immune synapse was likely forming between the CD8 T cells and neurons. However, undetectable levels of neuronal class I implies that a non-traditional engagement by CD8 T cells cannot be ruled out.

It was intriguing that in spite of many examples of TCRβ polarization, we never observed an instance in which CD3 protein bound to a CD8 T cell polarized against a target neuron. This was unexpected because CD3 is considered to be part of the TCR complex. Indeed, other studies report the presence of CD3 protein at immune synapses (2, 33). CD3 consists of 4 chains, γ, δ, ε, and ζ. These chains contain immunoreceptor tyrosine-based activation motifs which have been shown to be critical for both T cell activation and interaction with target cells. The role of ε and ζ are especially critical since they have been shown to have the capacity to transduce signals necessary for T cell activation even in the absence of other components of the TCR complex (8, 11). The antibody used in these studies was specifically against the ε chain. It may be that during CD8 T cell engagement of a neuron, the ε chain is excluded from the synapse. This may indicate that the immune synapse between a CD8 T cell and neuron is fundamentally different from synapses with other CNS cell types. Exclusion of the ε chain from the synapse may be a defense mechanism that neurons have evolved in order to avoid being killed by CD8 T cells. Indeed, at least one study has put forward a hypothesis utilizing this defense mechanism. Data from this work suggests that interaction with neurons may be able to change to phenotype of CD4 T cells from effector to suppressor (7). Alternatively, it is possible the ε chain is not needed to complete an interaction between a CD8 T cell and neuron. Perhaps the ζ chain is responsible for signaling an effector
function in this situation. Another possibility for lack of observed CD3 polarization is that different splice variants of the ε chain may be present at such a neuronal synapse making the commercial antibody used in these studies unable to bind to it. Alternatively, it may be possible that CD3 polarization is highly transient in a synapse between a CD8 T cell and neuron. If CD3 protein polarization is short lived, finding such an event would be increasingly difficult.

Consistent with the findings of other studies, we did not observe high levels of MHC class I protein expression on neurons (18, 20). One possible reason that neurons did not robustly label with MHC class I protein is that alternate splice variants may be expressed by this cell type. High levels of mRNA have been shown to be present within neurons but expression of class I protein is apparently very low (18, 44). This likely indicates that class I expression is very tightly controlled. Indeed, this is plausible since studies have shown that neurons only upregulate MHC class I expression under specific circumstances including during a vigorous immune response. Therefore, class I expression may be under the control of many regulatory mechanisms. Protein modification may be one such regulatory mechanism employed by neurons to keep class I levels low. Once robust expression of class I is needed such protein modification systems may allow for the translation of class I but ultimately alter the protein in some way, resulting in splice variants. Alternatively, it may be that the immune response to TMEV within the C57BL/6 mouse may not elicit upregulation of MHC class I expression. The addition of relatively high levels of IFN-γ to neuronal cultures has been shown to upregulate class I molecule expression (18, 30, 44). It is possible that the physiological IFN-γ levels present in the inflamed CNS of our mouse model is
insufficient to allow MHC class I upregulation. However, only low levels may be
necessary to form an immune synapse. Previous studies have demonstrated that T cells
have the ability to form immune synapses with target cells that have greatly reduced
MHC class I molecule expression (12, 13). Therefore, if not expressed at high levels,
MHC class I molecules may be abundant enough on neurons in our model to
accommodate immune synapse formation.

Traditionally, CD8 T cells may form immune synapses to perform effector
functions against a virally infected cell type (8, 23). At this synapse, granzyme B is
utilized by the CD8 T cell to initiate the caspase cascade that leads to apoptosis in the
target cell. Though, it has been shown that this is not always the case (21). In certain
viral models, CD8 T cells may utilize granzyme B to control viral infection within cells
rather than as a killing mechanism (21). Comparatively, within TMEV infected neurons
we observed indications of degeneration known as dendritic beading. This
morphological phenotype manifests as the replacement of intact neuronal processes with
bead-like structures. The soma of many infected neurons was also undergoing this
beading process as well. However, in our studies it remains unclear if CD8 T cell
engagement of neurons contributes to their degeneration. It has been shown that TMEV
infection alone can contribute to neuronal degeneration (40, 41). Therefore, whether
CD8 T cell engagement contributes to the degeneration of neurons in our model remains
a critical topic of research.

Observations presented here support a model in which CD8 T cells directly
interact with neurons via a traditional immune synapse in the TMEV infection model of
MS. Previous studies have demonstrated that axonal injury and loss are readily observed
in this model (14, 15, 40). Genetic deletion of CD8 T cells resulted in relative preservation of axons when compared to animals with intact CD8 T cell populations (15, 17). Additionally, removal of a select population of virus-specific CD8 T cells resulted in Therapeutic motor preservation of chronically infected animals. These data provided evidence that CD8 T cells were directly associated with neuronal pathology and clinical disability. However, there was no evidence demonstrating the direct interaction of CD8 T cells with neurons. Many studies have shown that little or no detectable expression of neuronal MHC class I, thought to be a prerequisite molecule for traditional immune synapse formation by a CD8 T cell (17, 18). On the contrary, studies have demonstrated that most types of glial cells readily express class I in vitro (31). Further, all major glial cell types have been shown to be susceptible to lytic attack by CD8 T cells (31). Taken together, these data have led to the dismissal of models in which CD8 T cells directly interact with neurons. Instead, this evidence popularized the hypothesis that CD8 T cells indirectly create neuronal pathology by attacking glial cells that surround neurons. In this indirect attack model, the effector action of CD8 T cells and the resulting activation of microglia create a toxic milieu that injures neighboring neurons. Evidence supporting the indirect CD8 T cell attack model has largely been derived from in vitro observations (31). Whereas indirect CD8 T cell attack may represent a potential mechanism by which neuronal death occurs, additional in vivo research is needed to clarify the role that this model may play in TMEV induced disease.

While not mutually exclusive from the indirect CD8 T cell attack model, data presented in these chapters provide strong in vivo evidence that supports a model in which CD8 T cells directly interact with neurons. In this model, CD8 T cells directly
engage a neuron via a traditional immune synapse as indicated by our observations of polarized TCRβ (Figure 12). Neurons are then potentially injured or killed by effector molecules as evidenced by our data showing polarized granzyme B. While this data is intriguing it does not define if granzyme B was being utilized to destroy neurons. To establish if CD8 T cells were performing an effector function in response to neuronal engagement it should be known if this granzyme B was being released and fully functional. CD104a labeling could be employed to demonstrate that granzyme B was being actively released by the CD8 T cell when engaging neurons (21). To determine if granzyme B was functional, granzyme B knockout mice on a C57BL/6 background could also be utilized. The adoptive transfer of CD8 T cells from wildtype C57BL/6 mice and CD8 T cells from the granzyme B knockout mouse into separate experimental groups of TMEV infected T cell deficient RAG-/- mice could provide a model to study the functionality of granzyme B. Assuming CD8 T cells interacted with neurons in this model, higher levels of neuronal death in RAG-/- animals with adoptively transferred wildtype CD8 T cells compared to RAG-/- animals with granzyme B-/- CD8 T cells would indicate CD8 T cells were likely performing an effector function. Alternatively, if neuronal death was lower in RAG-/- mice adoptively transferred with granzyme B competent CD8 T cells, this approach would provide evidence that this effector molecule was being used to control virus infection without killing the neuron as has been shown to occur under a specific virus infection. Equal levels of neuronal death between groups of RAG-/- mice receiving either granzyme B deficient or competent CD8 T cells could indicate that either CD8 T cells are not involved in contributing to neuronal death or a granzyme B independent effector mechanism is utilized.
Albeit clearly different potential mechanisms of neuronal pathology, both the direct and indirect models of CD8 T cell-induced neuronal pathology may work in concert within animals susceptible to chronic TMEV-induced disease (Figure 12). It is possible that within the first week of TMEV infection, as we observed, neuronal pathology occurs primarily by direct CD8 T cell engagement of infected neurons. At later time points, virus spreads to glial cell types which then likely become CD8 T cell targets. In turn, CD8 T cell attack on glial cells could create an environment with high levels of inflammatory cytokine expression that may be toxic to bystander neurons. This environment could then interrupt normal neuronal function or induce apoptosis.

CD8 T cell-mediated pathology is not the only proposed mechanism of neuronal death in the TMEV model. TMEV infection alone has been demonstrated to induce apoptosis in hippocampal neurons (41). Apoptosis among hippocampal neurons was observed as early as 2 days after infection and peaked 4 days after infection in C57BL/6 mice (9). These time points precede infiltration of CD8 T cells into the brain (27, 28). The C57BL/6 strain eventually clears TMEV infection, and because the apparent CD8 T cell-induced pathology is localized to non-motor areas of the CNS, this strain suffers no long term MS-like motor dysfunction (32). However, studies have clearly shown that sparing of axons and motor function occurs with the removal of CD8 T cells in certain strains of mice susceptible to chronic TMEV infection and disease (14, 15, 17, 42). It is likely that multiple mechanisms leading to neuronal death are present. Therefore, the extent that viral infection or CD8 T cell interaction contributes to neuronal pathology remains a critical topic of research.
Observations of direct CD8 T cell interaction with neurons may also be applied to human conditions. High levels of CD8 T cell CNS infiltration and clonal expansion is present within MS patients (1, 35). Further, a correlative relationship between CD8 T cell infiltration and neuronal injury has been established (5). Additional human diseases such as virus encephalomyelitis, paraneoplastic disorders and Rasmussen’s encephalomyelitis are all conditions in which CD8 T cells have been linked to neuronal pathology (4, 38). However, the mechanism utilized by CD8 T cells to potentially perpetuate neuronal injury in these disorders remains undefined. Data presented here provide strong evidence that supports a model in which CD8 T cells can directly interact with neurons via an immune synapse. This basic mechanism may be a common denominator in all conditions in which CD8 T cells contribute to neuronal pathology. Therefore, a treatment that is effective at counteracting CD8 T cell engagement of neurons could potentially be therapeutic for a wide variety of disease conditions.

Studies from these chapters provide strong evidence to support the hypothesis that CD8 T cells can engage neurons via an immune synapse. Further, it is likely that these CD8 T cells are performing an effector function when interacting with these neurons. Whether CD8 T cell engagement of neurons contributes to neuronal degeneration is a topic of ongoing research. However, this evidence provides a mechanism by which CD8 T cells can directly engage neurons. The direct engagement can potentially perpetuate neuronal pathology in a number of disease conditions. Finally, we provide novel evidence suggesting that TCEP morphology is associated with lymphocytic movement in the CNS comparable to T cell motility within lymph nodes.
References


Figure 12- Class I: peptide antigen specific CD8 T cells potentially damage axons through two mechanisms. (A) In the direct mechanism, CD8 T cells damage axons through direct recognition of class I:peptide epitopes on the surface of axons. (B) In the indirect mechanism, CD8 T cells potentially damage axons indirectly by killing glial cells that present the class I:peptide epitope, leaving axons exposed to other forms of inflammation. The shown is one example of an indirect mechanism where an oligodendrocyte presenting class I:peptide antigen to CD8 T cells is targeted.
Appendix
Intracerebral hemorrhage leads to infiltration of several leukocyte populations with concomitant pathophysiological changes**

**Portions of this work adapted with permission from:
Abstract

Intracerebral hemorrhage (ICH) is a stroke subtype with high rates of mortality and morbidity. The immune system, particularly complement and cytokine signaling, has been implicated in brain injury after ICH. However, the cellular immunology associated with ICH has been understudied. In this report, we use flow cytometry to quantitatively profile immune cell populations that infiltrate the brain 1 and 4 days post-ICH. At 1 day CD45^{hi} GR-1^{+} cells were increased 2.0-fold compared with saline controls ($P < 0.05$); however, we did not observe changes in any other cell populations analyzed. At 4 days ICH mice presented with a 2.4-fold increase in CD45^{hi} cells, a 1.9-fold increase in CD45^{hi} GR-1^{-} cells, a 3.4-fold increase in CD45^{hi} GR-1^{+} cells, and most notably, a 1.7-fold increase in CD4^{+} cells ($P < 0.05$ for all groups), compared with control mice. We did not observe changes in the numbers of CD8^{+} cells or CD45^{lo} GR-1^{-} cells ($P=0.43$ and 0.49, respectively). Thus, we have shown the first use of flow cytometry to analyze leukocyte infiltration in response to ICH. Our finding of a CD4 T-cell infiltrate is novel and suggests a role for the adaptive immune system in the response to ICH.
Introduction

Intracerebral hemorrhage (ICH), a bleeding into the brain parenchyma, can occur in neonates, children, or adults either spontaneously or as a result of trauma (14, 20). ICH is the stroke subtype with the highest rates of mortality and morbidity; only 38% of patients survive for 1 year (5, 20, 25). Breakdown of the blood-brain-barrier (BBB) is well documented in ICH, leading to extravasation of plasma proteins and marked perihematomal interstitial and vasogenic edema (17, 25).

There are numerous reports indicating that various immune components are associated with the hemorrhagic milieu after ICH, including proinflammatory cytokines, complement components, microglial activation, and neutrophil infiltration (7, 9, 22, 24, 25). However, there is very little work detailing the types of leukocytes that enter the brain after ICH. Furthermore, studies that do exist are largely limited to immunohistochemical techniques. A more precise assessment of these leukocytes, including determining the frequency of central nervous system (CNS) infiltrating immune cells, is needed to understand the role of the immune system in the pathogenesis of ICH-induced brain injury.

In this report, we are the first to use flow cytometry to quantitatively study the leukocytes that enter the brain after experimental ICH in mice. We report that ICH leads to an increase in immune cell populations, including CD4 T cells and total blood-derived leukocytes, with concomitant alterations in physiologic and behavioral parameters. These data suggest a role for CNS inflammation in ICH.
Results

To verify our ICH model, we fixed and sectioned some of the brains to inspect the hematoma. Figure 13A shows a representative brain, cut in 2 mm serial sections. Visual examination of the brain reveals a mild to moderate hemorrhage with the hematoma centered in the anterolateral portion of the left hemisphere extending into the striatum. There is also some observable perihematomal edema.

Figure 13B shows the results of whole-brain water content measurements, used as a marker of edema. At 4-day ICH, animals had a small, but statistically significant, increase in brain water content compared with saline controls (78.0 versus 77.7%; \( P < 0.05 \)). We did not observe increased brain water content at 1-day post-ICH. We used the rotarod task to assess motor deficits resulting from ICH (10). At 1 and 4 days the ICH group showed a poorer rotarod performance compared with saline controls (presurgery compared with immediately before euthanasia; \( P < 0.05 \)). These results are shown in Figure 13C. Lee et al. have also shown rotarod differences between ICH and control groups in a rat model of ICH (16).

An immune response after ICH in humans and animal models has been noted (6, 25-27). We wished to characterize the immune response in our ICH model 1 and 4 days post-ICH because important clinical sequelae begin to appear between 1 and 5 days post-ICH (20). Using flow cytometric analysis we have showed that similar leukocyte populations were present in the brains of both saline and ICH mice; however, the numbers specific inflammatory cell types differed in ICH mice as compared with saline
controls. At 1 day, we observed an increase in CD45$^{\text{hi}}$ GR-1$^+$ cells ($P < 0.05$) compared with saline controls, but did not find changes in CD45$^{\text{hi}}$ ($P = 0.11$), CD45$^{\text{lo}}$ GR-1$^-$ ($P = 0.46$), CD8$^+$ ($P = 0.32$), CD4$^+$ ($P = 0.13$), or CD45$^{\text{hi}}$ GR-1$^-$ ($P = 0.39$) cells. At 4 days, we did not observe any difference in CD45$^{\text{lo}}$ GR-1$^-$ ($P = 0.49$) or CD8$^+$ cell populations ($P = 0.43$) between ICH and saline groups. However, at 4 days, ICH mice had a statistically significant increase in CD45$^{\text{hi}}$ cells ($P < 0.05$), CD4$^+$ cells ($P < 0.05$), CD45$^{\text{hi}}$ GR-1$^-$ cells ($P < 0.05$), and CD45$^{\text{hi}}$ GR-1$^+$ cells ($P < 0.05$) over saline-treated mice.
Discussion

In this study, we have examined leukocyte trafficking into the CNS after ICH. Our goal was to use a quantitative method to define the inflammatory cell infiltrate after ICH and to associate this leukocyte infiltration with pathophysiological data. There is a paucity of flow cytometric data in the literature defining the specific inflammatory cell types that infiltrate into the parenchyma during brain inflammation. However, multiple sclerosis has been characterized with regard to leukocyte infiltration. In contrast to our findings with ICH, CD8⁺ T cells traffic into the brain, in addition to other cell types, during multiple sclerosis (11).

Leukocytes, particularly T cells, traffic into the CNS under normal physiologic conditions. However, under these conditions, cells rarely enter the brain parenchyma and do not remain in the CNS for long periods of time unless they experience antigen and become activated (2, 8, 21). Immune trafficking into the brain parenchyma as a part of the stroke pathologic assessment has gone relatively unstudied. A previous report has used histochemical and immunohistochemical methods to show the presence of neutrophils, microglia, and CD8a⁺ cells in the brain after experimental ICH in the rat (27). However, our use of flow cytometry extends these observations and enables one to rapidly identify and quantify these cell types, as well as their ratios to one another.

There are two possible sources of leukocytes found in the brain parenchyma after ICH. Some cells will migrate from the hematoma into the perihematomal tissue and others will enter through an open BBB (25). As noted previously, we observed similar populations, but different numbers of immune cells among ICH and saline mice. This is likely because of the fact that the saline treatment produces a small amount of brain
injury as a result of the needle tracks, microhemorrhages, and mass effect from the volume of the injection.

We chose 1 and 4 days time points for this analysis based on the amount of erythrocyte lysis and subsequent inflammatory stimuli. In rodent models of ICH, erythrocyte lysis is low at 1 day and high at 4 days (23). Because erythrocyte products may contribute to inflammation, this is a logical choice of time points. Our observation of increased T cells at 4 days but not 1 day suggests recruitment from the periphery as opposed to the hematoma.

A significant increase in CD45^{hi} GR-1^{+} cells at 1 and 4 days was observed in ICH mice compared with saline controls (Figures 14 and 15C). A CD45^{hi} GR-1^{+} cell population is mostly comprised of neutrophils. An increase in neutrophils is not surprising at these time points, as these cells are a part of the innate immune response and typically are among the first inflammatory cells to migrate to the site of injury (3). These cells perform pattern recognition responses as well as promote inflammation (3). Our observed CD45^{lo} GR-1^{-} profile is characteristic of a microglia population (18) (Figures 14 and 15B). Like neutrophils, microglia are part of the innate immune system. Similar numbers of microglia in ICH and saline mice suggest that this cell type does not proliferate specifically in response to ICH.

On analysis of T lymphocyte subsets, we did not observe a difference in CD8^{+} cells between ICH and saline mice at either time point (Figure 16C). Conversely, we observed a significant increase in CD4^{+} cells in ICH-treated mice at 4 days but not 1 day (Figure 16B). These CD4^{+} cells are likely to be CD4^{+} T cells as they stain positively for CD3 and express CD45^{hi} that is characteristic of this lymphocyte subset. CD4^{+} T cells are
part of the adaptive immune system and may take 5 days or more to become antigen specific in the CNS (1). Increased numbers of CD4+ T cells in ICH is interesting, as traditional helper T cells do not normally recognize syngeneic protein as antigen. This suggests that the increased numbers of CD4+ cells in ICH could be the result of bystander activation or the recognition of endogenous antigens that have become newly exposed to the immune system as a result of ICH. Another possibility is that this CD4+ cell population contains regulatory T cells. CD4+ regulatory T cells have been shown to recognize both foreign and syngeneic antigens (4, 15, 19). Therefore, it is currently unclear what functional role that the observed increase in CD4+ cells might play in the pathophysiology associated with ICH. However, there may be a chemospecific response occurring that is specific to the extravascular blood.

Using our model, at 4 days, we also observed a significant increase of CD45^hi GR-1^- cells (P<0.05) in ICH-treated mice as compared with saline controls (Figure 14D). More than 90% of this population is CD4 and CD8-, and remains uncharacterized in our flow cytometric analysis (data not shown). Although speculative, this population could contain macrophages, B cells, natural killer cells, as well as other blood-derived inflammatory cells. The role of these cell types remains unclear and will be the subject of future analysis in this model.

In summary, we have found infiltration of neutrophils into ICH brains at 1 day, and infiltration of several leukocyte populations at 4-day post-ICH. The observation that CD4+ T cells migrate into the brain in this murine model is significant because it implies a role for the adaptive immune system in ICH. This study therefore shows the need for
future research designed to adequately define the contribution of specific helper T-cell subsets in ICH.
Materials and methods

Unless otherwise noted, all materials and reagents were purchased from Sigma (St Louis, MO, USA).

Animals: All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC). Male C57BL/6J mice (approximately 20 to 30 g) were purchased from Jackson Laboratories (Bar Harbor, NE, USA). Animals were housed in standard laboratory housing and allowed ad libitum access to food and water.

ICH Model: Mice were anaesthetized with 2% isoflurane in 24% oxygen and 74% nitrous oxide, administered through an anesthesia mask (Kopf, Tujunga, CA, USA). Deep sedation was monitored throughout the procedure by the absence of pain reflexes in the toes and pupils. Body temperature was held at 37°C with a feedback-controlled heating blanket. Our experimental ICH procedure was based on a previously described method by Yang et al., with some modifications (28). Mice were placed in a stereotaxic frame (Kopf). A skin incision was made along the midline of the dorsal surface of the skull, exposing the bregma. A 1 mm cranial burr hole was drilled 2.5 mm lateral and 0.5 mm anterior to the bregma. Approximately 30 µL of autologous blood was collected by clipping a small portion of the distal tail. Further bleeding was prevented by cauterization of the tail. Blood was drawn into a 50 µL Hamilton syringe with a 26-gauge needle. The needle was inserted 4 mm ventral through the cranial hole into the brain and 20 µL of blood or sterile saline was infused more than 15 mins. After infusion, the hole in the skull was filled with dental cement and the incision was closed. Mice were allowed to survive
for 1- or 4-days post-ICH, at which time they were euthanized with isoflurane and decapitation.

Brain Water Content Measurement: Mice were euthanized and brains were removed and immediately weighed on an analytical balance. Brains were desiccated by heating at 85°C for 16 h and weighed again. Whole-brain water content was measured as a marker of edema. Brain water content was calculated according to the following formula: Brain water content = (wet weight - dry weight) / wet weight (28).

Motor Deficit: Motor deficits were assessed using the rotarod task. The amount of time mice were able to stay on the top of the rotating rod (32 r.p.m., constant speed) was measured before surgery and again before euthanasia. The percent change from presurgery to the time before euthanasia was calculated. This value was used in the comparison between the blood infused and saline-infused groups.

Isolation of Central Nervous System Immunologic Cells: Cells were isolated as described previously (13). Briefly, excised mouse brains were pushed through a nylon cell strainer (BD Biosciences, Franklin Lakes, NJ, USA) with a 100-µm pore width and incubated at 37°C for 45 mins in 250 µg/mL collagenase type 4 (Worthington Biochemical Corp., Lakewood NJ, USA). Immunologic cells were then concentrated via continuous Percoll gradient (Pharmacia, Piscataway, NJ, USA) centrifugation at 10,000 r.p.m. (Sorvall SS-34 rotor) for 30 mins. The resulting layer of immunologic cells was then collected and placed into a 50 mL falcon tube. RPMI (1X) was added until the final volume was 50 mL and the solution was then centrifuged at 1500 r.p.m. (Sorvall Legend RC centrifuge) for 10 mins. The resulting cell pellet was resuspended in fluorescence-activated cell sorting
buffer (1% bovine serum albumin and 2% sodium azide). It is important to note that the hematoma was filtered out of cell suspensions before flow cytometric analysis. Therefore, flow cytometric analysis was not influenced by cells potentially residing within the hematoma itself. Further, it is unlikely that cells would remain viable 1 or 4 days post-ICH within a blood clot. Thus the cell populations observed are highly likely to only be leukocytes that infiltrated into the parenchyma from the periphery or from the hematoma during retraction of the clot.

**Flow Cytometric Analysis:** Cells isolated from the CNS were stained with anti-CD4 PE, anti-CD8 FITC, anti-CD3 APC, anti-CD45 PE-Cy7, and anti-Gr-1 APC-Cy7 on ice for 45 mins. Samples were then washed twice with fluorescence-activated cell sorting buffer, resuspended in cold phosphate-buffered saline, and fixed in 1% paraformaldehyde. Samples were then analyzed on a BD LSRII instrument (BD Biosciences). Raw data were displayed with side scatter along the $y$ axis and forward scatter along the $x$ axis. A gate was placed according to the profile of inflammatory cells as previously determined (11, 12). This parent population was then examined on a scatterplot with CD45 expression on the $y$ axis and GR-1 expression on the $x$ axis as shown in Figure 2. Cell populations that expressed CD45 at $10^3$ channels or higher and CD3 at $10^{2.5}$ channels or higher were considered a population of interest and gated for analysis. Cells that expressed the CD4 cell-surface marker at $10^3$ channels or higher and showed a side scatter of 40,000 or less were selectively gated and then plotted. Likewise, cells that expressed the CD8 cell-surface marker at $10^3$ channels or higher and showed a side scatter of 40,000 or less were selectively gated and then plotted. Using flow cytometric analysis of brain-isolated inflammatory cells, we determined that similar ratios of
leukocyte populations were present in the brains of both ICH and saline-treated mice. Therefore, absolute numbers of inflammatory cells were considered to perform statistical analyses.

*Statistical Analysis:* Data were analyzed with the statistical package in Microsoft Excel using Student's *t*-test or a paired *t*-test. The level of significance was $< 0.05$. 
References


Acknowledgements

This work was supported in part by a grant from the NIH: R01NS050569 (JFC).
Figure 13- Physiologic and behavior changes associated with intracerebral hemorrhage (ICH). (A) ICH produced a mild to moderate hematoma in the anterolateral portion of the brain near the striatum. (B) ICH led to increased edema at 4 days when compared with saline-treated mice (n=4 to 6 per group, P<0.05). Edema was measured as total brain water content. (C) ICH showed poorer motor function at 1 and 4 days compared with saline controls (baseline versus 1 or 4 days; n=6 to 7 per group, P<0.05). Bars are mean±s.e.m.; *P<0.05 compared with saline of the same time point.
Figure 14- Representative scatter plots showing analysis of CD45hi cells at 4 days in a mouse receiving intracerebral hemorrhage (ICH) and a saline control. An overall increase in CD45hi inflammatory cell infiltrate was observed at 4 days post-ICH, compared with saline-treated mice (P<0.05), but not at 1 day (P=0.11). Cells were considered to be CD45hi if they expressed CD45 at 103.2 channels or higher. These cells were then gated and counted for statistical analysis. Gate P2 is considered to be CD45hi GR-1+, gate P3 is considered to be CD45hi GR-1+, and gate P4 is considered to be CD45lo GR-1+. 
Figure 15- Assessment of blood-derived inflammatory cells in the CNS 1 and 4 days after intracerebral hemorrhage (ICH). Brain infiltrating inflammatory cells were stained with anti-CD45, a pan marker for blood-derived cells, and anti-GR-1 antibodies. GR-1 is a pan surface marker for granulocytes. (A) At 4 days there were an increased number of blood-derived leukocytes in ICH brains compared with saline-treated brains (P<0.05); this was not observed at 1 day (P=0.11). (B) We did not observe a difference in CD45lo GR-1+ cells (microglia) between ICH and saline mice at 1 or 4 days (P=0.46 and P=0.49, respectively). Conversely, we found an increased CD45hi GR-1+ cell population (neutrophils) at 1 and 4 days (C) (P<0.05) and an increased CD45hi GR-1+ cell population (cells of the lymphoid lineage and macrophages) at 4 days, but not 1 day (D) (P<0.05 and P=0.39). N=3 to 4 per group; bars are mean±s.e.m.; *P<0.05 compared with saline of the same time point.
Figure 16 - CD4+ and CD8+ cells. (A) Representative scatter plots showing flow cytometric analysis of CD4+ cells in intracerebral hemorrhage (ICH) and saline brains at 4 days. (B) We observed increased numbers of CD4+ cells in ICH mice compared with saline controls (P<0.05) at 4 days but not at 1 day (P=0.13; C) There was not a difference in CD8+ cells between ICH and saline mice at 1- or 4-day post-ICH (P=0.32 and P=0.43, respectively). N=3 to 4 per group; bars are mean±s.e.m.; *P<0.05 compared with saline of the same time point.
University of Cincinnati
Institutional Animal Care and Use Committee

Protocol Modification Approval Notification

Principal Investigator(s): Aaron Johnson, Ph.D and Kristina Bielewicz B.S

Protocol Action: Modification  Protocol #: 06-10-09-01

Protocol Title: CD8 Mediated Neuropathology in theiler's Virus Infection

Approval Date: 11/9/06  Modification Approval Date: 11/24/08

Expiration Date: 11/9/09

- Protocols can only be approved for a three-year period. A Protocol Update must be submitted to renew the protocol. The IACUC Office recommends submitting an Update three months prior to the expiration date of the protocol.

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- If this protocol has been issued a new number to replace the previous number, all identification cards in animal housing facilities must be changed to reflect the new protocol number.

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3rd August 2009

Dear Jeremiah,

RE:  McDole J, Johnson AJ, Pirko I. “The role of CD8+ T-cells in lesion formation and axonal dysfunction in multiple sclerosis” Neurological Research. 2006 Apr;28(3):256-61 specifically the following sections only: the Introduction, the first three paragraphs from the section entitled “CD8 T-cells in MS”, the entire section entitled “Evidence for CD8 T-cell mediated axonal loss” and all of figure 1.

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| Portion | Full article |
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Publications  
McDole J., Danzer, S., Pun, R. Y., Pirko, I., Johnson, A.J. “Rapid formation extended processes and engagement of neurons by CNS-infiltrating CD8 T cells.” (manuscript in prep)  


Abstracts


Techniques

Densitometry, PCR/RT-PCR, primer design, DNA/RNA isolation, plasmid insertion, ELISA (IFN-g), CNS cell culture, cryostat sectioning of fresh frozen tissue, immunohistochemistry, confocal microscopy, fluorescence activated cell sorting (FACS), lymphocyte isolation, intracranial infection, perfusion, behavioral assays (water mazes, rotarod), mouse breeding.

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