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Short Communication

ICAM-1 is crucial for protection from TMEV-induced neuronal damage but not demyelination

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Previous work has suggested that the factors protecting mice from Theiler's murine encephalomyelitis virus (TMEV)-induced spinal cord demyelination are distinct from those involved in protection of the brain during the acute encephalitic phase. In this study, we examined the requirement for intercellular adhesion molecule-1 (ICAM-1) in both of these processes. During the acute phase of infection (days 7 to 10 after intracerebral infection with TMEV), no differences in brain or spinal cord pathology or virus burdens were observed between ICAM-1–knockout mice and the infected immunocompetent control mice of a similar background. Examination of brain pathology later in infection (that is, day 45 post infection [p.i.]) revealed that ICAM-1–deficient mice experienced increased levels of pathology in gray matter regions of the brain. We observed an increase in striatal damage and meningeal inflammation in the brains of TMEV-infected ICAM-1–knockout mice compared to C57BL/6J mice. Despite the increase in brain pathology, no immunoreactivity to viral antigens was detected, suggesting that the virus had been cleared by this time. Resistance to demyelination was similar in both groups, indicating that the resulting immune response was sufficient for protection of the spinal cord white matter. *Journal of NeuroVirology* (2002) **8,** 452–458.

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Under normal conditions, minimal adhesion molecule expression is detected in the central nervous system (CNS). Following infection, injury, or induction of autoimmune disease, the host rapidly upregulates intercellular adhesion molecule-1 (ICAM-1) in the CNS (Raine and Cannella, 1992; Kraus *et al*, 1998; Mycko *et al*, 1998; McDonnell *et al*, 1999). ICAM-1 expression on endothelial cells is integral to lymphocyte migration (Reiss and Engelhardt, 1999). CNS resident cells such as astrocytes and microglia (Sobel *et al*, 1990; Shrikant *et al*, 1994; Drescher and Whittum-Hudson, 1996) also upregulate ICAM-1 during inflammatory reactions. In these cells, ICAM-1 is presumed to facilitate antigen presentation by interacting with leukocyte functional antigen (LFA-1) on T lymphocytes. Although the ability of the host to modulate expression of ICAM-1 may provide a protective advantage to the host, inappropriate expression of adhesion molecules may contribute (or be associated with) to irreversible pathology in sites such as the CNS (Raine and Cannella, 1992; Kraus *et al*, 1998; Mycko *et al*, 1998; McDonnell *et al*, 1999). *In vitro*, expression of ICAM is enhanced by proinflammatory soluble mediators such as tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), and interleukin (IL)-1β (Satoh *et al*, 1991; Shrikant *et al*, 1994; Hery *et al*, 1995; Drescher and Whittum-Hudson, 1996; Kyrkanides *et al*, 1999), suggesting one mechanism by which this molecule may be up-regulated during inflammatory processes.

Several investigators have suggested that ICAM-1 is a participant in the development of pathology in multiple sclerosis (MS), the most common demyelinating disease of humans (Kraus *et al*, 1998;

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Mycko *et al*, 1998; McDonnell *et al*, 1999). ICAM-1 expression is increased on blood vessels at the edges of active plaques, which would allow for extravasation of lymphocytes into the CNS (Kraus *et al*, 1998). In addition, some studies have found elevations in soluble ICAM-1 levels in the serum and/or cerebrospinal fluid of MS patients (McDonnell *et al*, 1999). More recently, genetic studies have identified an increased frequency of an ICAM-1 gene polymorphism in exon 6 of MS patients (Mycko *et al*, 1998). Taken together, these reports implicate ICAM-1 as a participant in the development or exacerbation of pathology in demyelinating disease.

Intracerebral infection of Theiler's murine encephalomyelitis virus (TMEV) induces a biphasic disease in susceptible mouse strains (Lipton, 1975). In the first 2 weeks after infection, virus replicates primarily in neurons, resulting in an acute encephalitis in the mouse (Dal Canto and Lipton, 1982). Following resolution of the acute phase, a chronic progressive, immune-mediated demyelinating disease occurs in susceptible mice. The spinal cord pathology induced is similar to that observed in MS (Dal Canto and Lipton, 1982). Because of the numerous studies focussing on the role of specific immune system components in protection from demyelination, this model has been used largely as a model of MS (Lipton and Dal Canto, 1976; Rodriguez *et al*, 1986, 1988, 1990a; Cash *et al*, 1989; Cash and Bandeira, 1991). More recently, studies have used this model to examine the discrete components of the immune system during the acute encephalitic phase of the disease (Drescher *et al*, 1999, 2000). In this study, we used mice genetically deficient in ICAM-1 (ICAM-1 $-/-$ mice) to assess the contribution of ICAM-1–mediated interactions to protection from TMEV-induced brain disease and demyelination.

At 4 to 6 weeks of age, ICAM-1–knockout mice (Sligh *et al*, 1993) on a background resistant to TMEV-induced demyelination ($H\text{-}2^b$), C57BL/6J (resistant to chronic infection/demyelination), B10.M, and B10.Q (both strains susceptible to chronic infection/demyelination), were intracerebrally infected with 2×10^5 plaque-forming units (PFU) of the Daniel's strain of TMEV in a total volume of 10 μl (Lipton and Dal Canto, 1979). Handling of the animals conformed to both the guidelines of the National Institutes of Health and the Mayo Clinic/Foundation and Creighton University Institutional Animal Care and Use Committees. At various times post infection (p.i.), mice were sacrificed with an overdose of pentobarbitol (Sleepaway; Fort Dodge Animal Health, Fort Dodge, IA) perfused with Trump's fixative. Brain and spinal cord blocks were prepared as previously described (Drescher *et al*, 1999, 2000). Additional mice were sacrificed and embedded in OCT (Elkhardt, IN), frozen sections prepared or samples prepared for viral plaque assays.

Immunohistochemical staining of frozen sections with a rat anti-mouse CD54 (ICAM-1) monoclonal antibody (BD Pharmingen, San Diego, CA) was performed per our previously published methodology (Drescher *et al*, 2000) to assess the extent of ICAM-1 expression in the brains and spinal cords of mice resistant (C57BL/6J) and susceptible (B10.M and B10.Q) to TMEV-induced demyelination over the course of infection. Regardless of their susceptibility to demyelination, minimal ICAM-1 expression was observed in the CNS of uninfected mice (Figure 1**A**). By day 3 p.i., ICAM-1 was up-regulated in the brains of the mice, with intense staining of the blood vessels and the choroid plexus (Figure 1**B**). These findings are consistent with those observed following infection with Semliki Forest virus and in experimental autoimmune encephalomyelitis (Samoilova *et al*, 1998; Smith *et al*, 2000). In these studies, insult (either viral infection or the induction of autoimmunity) demonstrated that ICAM-1 levels were rapidly induced in the CNS of the mice. To determine if the observed ICAM-1 up-regulation in our studies was dependent on virus exposure or could be induced by nonspecific short-term inflammation, we injected C57BL/6J mice with either TMEV or Hanks' balanced salt solution (HBSS, vehicle control). Immunohistochemical staining demonstrated that at this time point, brains from HBSS-injected mice were devoid

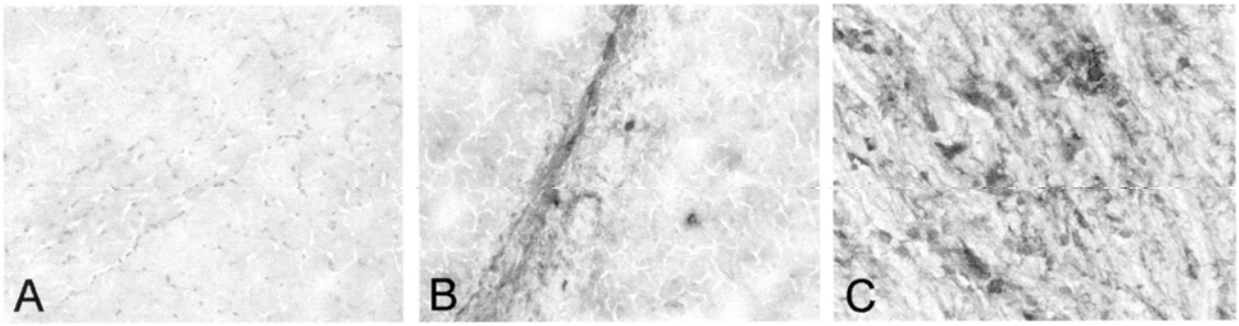

Figure 1 Expression of ICAM-1–positive cells in the brain and spinal cord of mice after infection with Theiler's virus. Minimal staining was observed in the brain of an uninfected C57BL/6J mouse (**A**). ICAM-1 expression is upregulated on blood vessels of the brain of a C57BL/6J mouse by day 3 p.i. (**B**). Immunoreactivity was also found in the spinal cord of mice susceptible to demyelination (B10.M) by day 21 p.i. (**C**). Frozen sections were stained using the immunoperoxidase technique with a monoclonal antibody specific to ICAM-1 per our published methodology (Drescher *et al*, 1998). The dark reaction product indicates positive staining.

of ICAM-1 signal, indicating that transient, low-level, nonspecific inflammation was insufficient for ICAM-1 expression at this time point (data not shown). We cannot rule out that there was brief ICAM-1 up-regulation in the brains of HBSS-injected mice at an earlier time point. We can, however, conclude that transient inflammation is not sufficient for sustained ICAM-1 expression. Some studies have identified certain cytokines (i.e., IFN-γ, TNF-α) that can directly induce or up-regulate ICAM-1 expression on CNS resident cells (Shrikant *et al*, 1994; Drescher and Whittum-Hudson, 1996). ICAM-1 expression in this model was not dependent on either TNF-α or IFN-γ, as intense ICAM-1 immunoreactivity was observed in the brains of TMEV-infected TNF-α–deficient (Pfeffer *et al*, 1993) and IFN-γ–deficient (Huang *et al*, 1993) mice on an *H-2*^{b}-background (data not shown) at day 5 after infection with TMEV.

By day 21 p.i., intense ICAM-1 staining was detected in the spinal cords lesions of TMEV-infected B10.M and B10.Q mice (susceptible to chronic infection; Figure 1**C**) and remained strong through at least day 45 p.i. (length of experiment). Minimal ICAM-1 staining was observed in the spinal cords of TMEV-infected C57BL/6J mice (resistant to chronic infection) at day 21 p.i. Minimal reactivity to the ICAM-1 antibody was detected in the spinal cords of uninfected mice, regardless of their susceptibility to demyelination.

In this model, all mice experience acute encephalitis regardless of their susceptibility to demyelination (Lipton, 1975), indicating the mechanisms involved in the genesis of pathology are unique in each of these two phases of the disease. Brain pathology was assessed in TMEV-infected and ICAM-1–knockout and C57BL/6J mice during the acute encephalitic phase (days 7 to 10). In addition, we also scored brain disease at a later time point. The day 45 p.i. time point was chosen as it is a classic time point when mice susceptible or resistant to spinal cord demyelination can be easily distinguished. By 45 days, the pathology in the brain has mostly disappeared (Rodriguez *et al*, 1990b, 1991). Brain pathology was scored using our previously published technique (Drescher *et al*, 1999, 2000). Pathologic scores were assigned to the resulting hematoxylin and eosin–stained slides without knowledge of experimental group. Each area of the brain was graded on a scale of 0 to 4 as follows: 0 = no pathology; 1 = no tissue destruction but only minimal inflammation; 2 = early tissue destruction (loss of architecture) and moderate inflammation; 3 = definite tissue destruction (demyelination, parenchymal damage, cell death, neurophagia, neuronal vacuolation); 4 = necrosis (complete loss of all tissue elements with associated cellular debris). Meningeal inflammation was assessed and graded as follows: 0 = no inflammation; 1 = one cell layer of inflammation; 2 = two cell layers of inflammation; 3 = three cell layers of inflammation; 4 = four or more cell layers of inflammation. The area with maximal extent of tissue damage was used for assessment of each brain region.

At 7 to 10 days after TMEV infection, a time when virus replicates primarily in the neurons of the brain, ICAM-1 −/− mice experienced severe disease in the cortex, hippocampus, striatum, and corpus callosum (Figure 2**A**). Despite this level of disease,

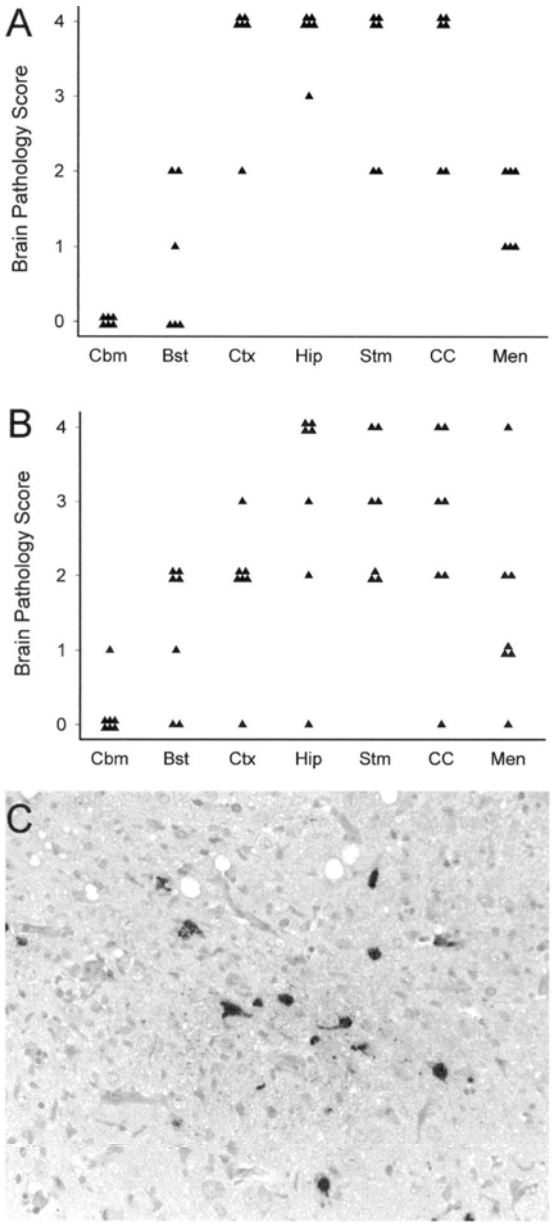

Figure 2 Brain disease in *H-2*^{b}-ICAM-1–knockout (**A, C**) and C57BL/6J (**B**) mice at 7 to 10 days post infection with TMEV. Disease scores are shown for the cerebellum (Cbm), brainstem (Bst), cortex (Ctx), hippocampus (Hip), striatum (Stm), corpus callosum (CC), and meninges (Men). Each symbol represents an individual mouse graded at each area of the brain according to the scale detailed in the methods. At days 7 to 10 post infection, severe disease in both the ICAM-1 −/− (**A**) and C57BL/6J mice (**B**) occurs. During the acute phase of TMEV infection, high levels of viral antigen were detected throughout the brains of the ICAM-1–deficient mice using our immunoperoxidase technique.

ICAM-1 −/− mice did not experience significantly more brain pathology than TMEV-infected C57BL/6J mice on the same genetic background (Figure 2**B**). Using a polyclonal antisera to TMEV, we also detected strong immunoreactivity to TMEV antigens throughout these brain regions in the ICAM-1 −/− mice (Figure 2**C**). To quantitate replicating virus in the central nervous system, we performed viral plaque assays on tissue obtained from animals sacrificed at day 7 p.i. ICAM-1 −/− mice did not experience increased TMEV burdens compared to control infected mice of the same genetic background (8.5×10^4 PFU/g CNS tissue). These data indicate that despite the absence of ICAM-1, these mice retained the capacity to control the viral load and damage to the levels observed in immune competent infected mice on the same genetic background.

Minimal pathology was observed in the brains of resistant C57BL/6J mice at day 45 p.i., an expected finding given that at this time point residual damage from the early phase of disease has resolved. The ICAM-1–knockout mice experienced increased levels of meningeal inflammation ($P < .01$, Mann-Whitney rank sum test) compared to the immunocompetent control infected mice of identical genetic background (Figure 3**A**, **B**, **C**). In addition to the increase in meningeal inflammation, ICAM-1–knockout mice also experienced severe striatal disease compared to the controls ($P < .01$, Mann-Whitney rank sum test). In 7 of the 10 ICAM-1 −/− mice examined, parenchymal disease characterized by areas of necrosis was observed. Presumably, the early pathology that was observed was not repaired. In contrast, no necrosis was observed in the brains

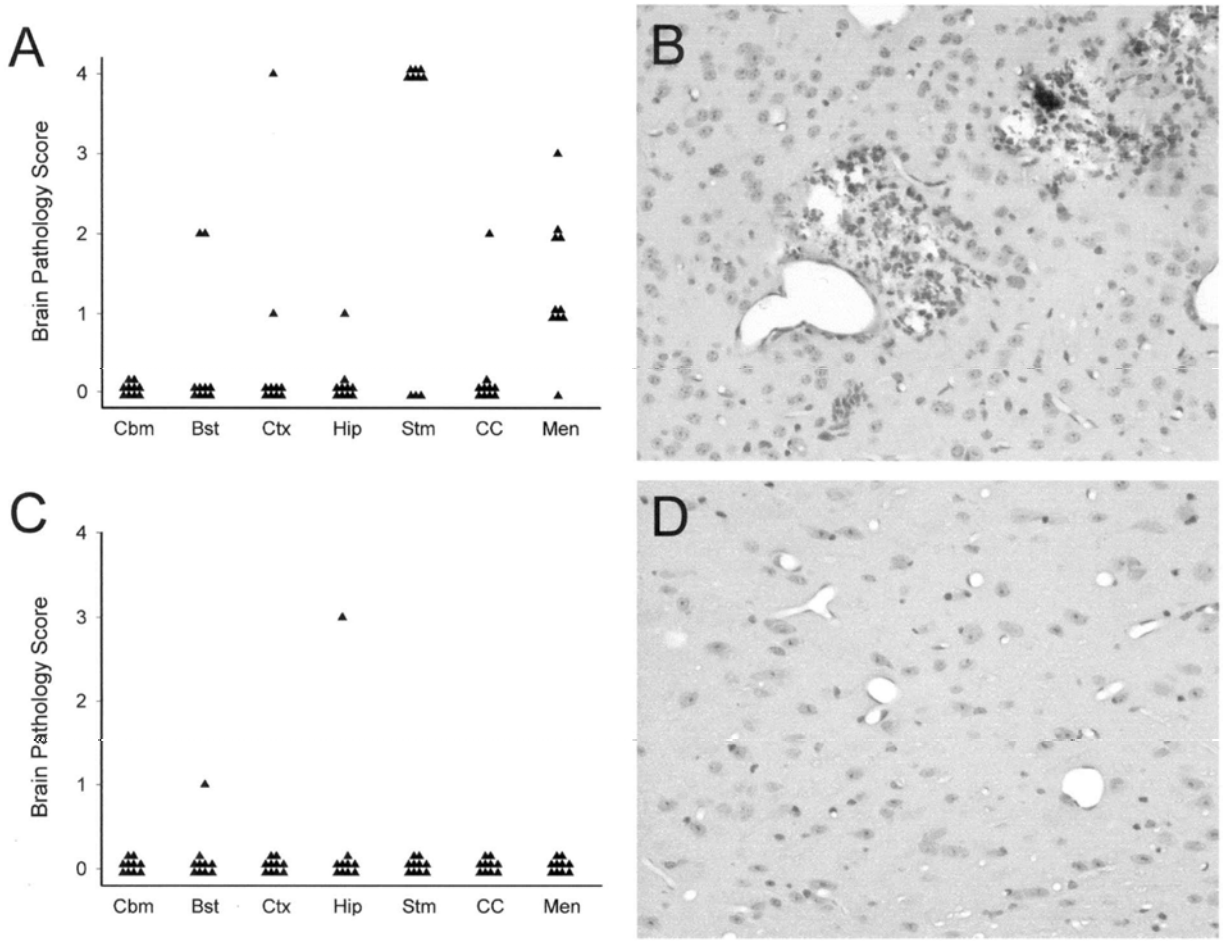

Figure 3 Brain disease in *H-2*^{b}-ICAM-1–knockout (**A**, **B**) and C57BL/6J (**C**, **D**) mice at 45 days post infection with TMEV. Disease scores are shown for the cerebellum (Cbm), brainstem (Bst), cortex (Ctx), hippocampus (Hip), striatum (Stm), corpus callosum (CC), and meninges (Men). Each symbol represents an individual mouse graded at each area of the brain according to the scale detailed in the methods. At day 45 p.i., ICAM-1–deficient mice (**A**, **B**) had significantly more disease in the striatum and meninges compared to infected C57BL/6J mice (**C**, **D**), as determined by the Mann-Whitney rank sum test. Examples of brain pathology are shown in (**B**) and (**D**). Severe striatal disease was observed in an TMEV-infected *H-2*^{b}-ICAM-1–knockout mouse (**A**) compared to an infected C57BL/6J mouse at day 45 p.i. (**C**). The striatum of the mouse in (**B**) was graded as a '4' due to the presence of necrotic tissue. The striatum of an infected C57BL/6J mouse is shown in (**D**) and graded as '0' due to the lack of inflammation and parenchymal damage. Paraffin-embedded sections were stained with hemotoxylin and eosin.

of the C57BL/6J mice. In the 10 C57BL/6J mice examined, only 1 had any brain pathology noted. In addition, moderate levels of meningeal inflammation of the spinal cords were observed in 9 out of the 10 the ICAM-1–knockout mice, whereas only 1 C57BL/6J mice experienced meningeal inflammation in the spinal cord.

Several studies have demonstrated that mice on a background resistant to TMEV-induced demyelinating disease become susceptible to demyelination if specific immune system components are suppressed either through the administration of blocking antibodies (Rodriguez *et al*, 1986, 1990a; Rodriguez and Sriram, 1988) or through genetic deletion (Fiette *et al*, 1993, 1995, 1996; Njenga *et al*, 1996; Drescher *et al*, 2000). To establish whether ICAM-1–dependent immune interactions are critical to protection from TMEV-induced spinal cord demyelination, we infected *H-2*^{b}-ICAM-1 −/− mice with TMEV and assessed the levels of gray matter inflammation, meningeal inflammation, and demyelination early and late in infection. Morphologic analysis was performed on 10 to 15 coronal sections per mouse. A pathologic score reflecting the frequency of pathology was assigned to each animal based on the presence of meningeal inflammation, neuronal inflammation, and demyelination. All scores were assigned without knowledge of genotype or treatment. The score was expressed as a percentage of the total number of spinal cord quadrants positive for a particular pathologic feature divided by the total number of spinal cord quadrants examined (Patick *et al*, 1990) according to our standard protocol. Plastic-embeddded spinal cord sections were prepared and stained with a modified erichrome stain with a cresyl violet counterstain (Pierce and Rodriguez, 1989). Spinal cords were examined both early (7 to 10 days p.i.) and late after TMEV infection. We chose day 45 p.i., as this time point has been previously found to differentiate between strains susceptible and resistant to TMEV-induced demyelination (Rodriguez *et al*, 1990b, 1991). Early after infection, both ICAM-1 −/− and C57BL/6J mice experienced moderate levels of meningeal and gray matter inflammation (Table 1). The presence or absence of ICAM-1 did not significantly impact the level of pathology. The combined data examining brain pathology, viral load, and spinal cord pathology early after TMEV infection supports the theory that ICAM-1 −/− mice retain sufficient immune processes to protect the CNS from overwhelming viral replication and damage.

By day 45 p.i., differences in spinal cord pathology were noted between the infected control mice and their ICAM-1–deficient counterparts. This increase in meningeal inflammation in both the brain and the spinal cord is not suprising, as ICAM-1 is a key regulator of leukocyte extravasation. Previous studies have demonstrated a close correlation between virus persistence and demyelination (Njenga *et al*, 1996). Based on the low level of demyelination observed in the ICAM-1–deficient mice, we predicted that minimal levels of TMEV would be detected in the spinal cord white matter of these animals. Immunostaining of the spinal cords of ICAM-1–knockout mice with a polyclonal antisera to TMEV demonstrated undetectable levels of viral antigen in both the gray and white matter of both the brain and the spinal cord (data not shown). Together, these data indicate that sufficient immune responses to TMEV are generated in these mice to control widespread virus replication in the CNS.

Our data are consistent with *in vivo* depletion studies by Rose *et al* (1999), which demonstrated no alteration in the level of demyelination following administration of an anti–ICAM-1 antibody to TMEV-infected SJL/J mice (susceptible to demyelination). Our data, along with those of Rose *et al*, indicate that ICAM-1 plays a minimal role in either the protection from or development of demyelination in either susceptible or resistant mice. In contrast with the findings of Rose *et al* (1999), we observed an increase in brain pathology in ICAM-1–deficient mice (Figures 2, 3). This difference in brain pathology reported between the two studies is likely attributed to the timing of the ICAM-1 depletion. In the depletion study, anti–ICAM-1 antibody was administered between days 21 and 28 p.i. By this time after infection, the acute encephalitic phase of the disease has resolved and pathology is normally limited to the brain stem and the spinal cord of SJL/J mice. In our study, the affected brain regions at the day 45 p.i. time point were primarily composed of gray matter. Although

Table 1 ICAM-1–knockout mice have increased meningeal inflammation following Theiler's virus infection

		Mean pathology score ± SEM		
Strain	n	*Gray matter inflammation*	*Meningeal inflammation*	*Demyelination*
ICAM-1 −/− (days 7–10)	6	13.0 ± 4.6	5.1 ± 1.5	0.8 ± 0.0
C57BL/6J (days 7–10)	7	20.8 ± 3.2	7.4 ± 1.3	0.0 ± 0.0
ICAM-1 −/− (day 45)	10	0.0 ± 0.0	4.3 ± 1.1*	0.5 ± 0.3
C57BL/6J (day 45)	10	0.0 ± 0.0	1.0 ± 0.7	0.9 ± 0.6

Note. For each mouse, 10 to 15 spinal cord sections were graded for gray matter inflammation, meningeal inflammation, and demyelination early (days 7 to 10) and late (days 45) in the infection. The data are expressed as the percentage of spinal cord quadrants with disease (mean ± SEM). n = number of mice. Differences between meningeal inflammation scores were statistically significant between C57BL/6 and ICAM-1–knockout mice ($P < .05$) at day 45 p.i., as determined using the Student's t test.

the precise reason for the exacerbated parenchymal damage has not been elucidated, there are several explanations that can be discarded. The similar virus burdens and pathology early in infection do not support a role for unrestrained TMEV replication in these animals. Gray matter regions, consisting primarily of neurons, are particularly vulnerable during the acute phase of the disease. One possible reason for the observed brain pathology in the ICAM-1 −/− mice is that despite similar viral loads during the acute phase of disease, the kinetics of TMEV clearance is altered, and the ICAM-1–deficient mice are less efficient at this process. Alternatively, it may be that ICAM-1–dependent processes are involved in repairing the brain after the virus has been cleared.

Numerous studies in a variety of CNS disease models have associated ICAM-1 expression with pathology (Kraus *et al*, 1998; Mycko *et al*, 1998; Samoilova *et al*, 1998; McDonnell *et al*, 1999; Smith *et al*, 2000), and the absence of ICAM-1 with disease resistance (Favre *et al*, 1999). Overall, the data presented in this paper support the hypothesis that although ICAM-1 is up-regulated in the brain and spinal cord following TMEV infection, this molecule is involved in immune or repair processes induced in the host in response to insult, as opposed to being involved in the development of pathology. The data also provide additional evidence for the hypothesis that the processes involved in protection from acute encephalitis are distinct from those involved in protection from demyelinating disease in the TMEV model of CNS infection. Further studies to identify these factors are underway.

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