



## Virus-induced CD8 + T cells accelerate the onset of experimental autoimmune encephalomyelitis: Implications for how viral infections might trigger multiple sclerosis exacerbations

Emily K. Rainey-Barger, Penelope K. Blakely, Amanda K. Huber, Benjamin M. Segal, David N. Irani\*

Department of Neurology, University of Michigan Medical School, 4007 Biomedical Sciences Research Building, 109 Zina Pitcher Place, Ann Arbor, MI 48109-2200, United States

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### ABSTRACT

Viral infections can exacerbate multiple sclerosis (MS) through poorly defined mechanisms. We developed an experimental system whereby infection with an asymptomatic neurotropic alphavirus caused a transient acceleration of experimental autoimmune encephalomyelitis (EAE) without altering the expansion or differentiation of autoreactive CD4 + T cells. Instead, this effect on the clinical course of EAE depended on CD8 + T cells that neither participate in viral clearance nor induce neuropathology in infected mice without EAE. Our system should be useful to further unravel how certain viral infections trigger MS exacerbations and to understand how CD8 + T cells can exert pathogenic effects within active demyelinating lesions.

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### 1. Introduction

Disease exacerbations in patients with relapsing–remitting multiple sclerosis (RRMS) mostly occur without any obvious precipitating event. These events, however, happen two- to three-times more commonly during or immediately following a viral infection (Sibley et al., 1985; Andersen et al., 1993; De Keyser et al., 1998; Edwards et al., 1998; Kriesel et al., 2004; Correale et al., 2006). Features of virus-associated multiple sclerosis (MS) exacerbations include close temporal proximity of the clinical attack to the infection (within a few weeks), infections causing fever, and infections lasting longer than five days (Sibley et al., 1985; Andersen et al., 1993; De Keyser et al., 1998). Determining how external stimuli trigger MS exacerbations has important clinical relevance; measures to prevent or mitigate such factors could ameliorate the longitudinal course of disease. Current disease-modifying therapies at best only partially reduce the frequency of MS attacks, further emphasizing the importance of elucidating how environmental triggers contribute to the pathogenesis of RRMS.

Emerging data suggest that CD8 + T cells may be important mediators of CNS damage during MS relapses. Detailed immunohistochemical studies of MS lesions reveal that substantial numbers of CD8 + T cells accumulate in active plaques, typically outnumbering CD4 + T cells (Liblau et al., 2002; Neumann et al., 2002; Junker et al., 2007). These central nervous system (CNS)-infiltrating CD8 + T cells exhibit

characteristics of oligoclonal expansion, a likely consequence of local, antigen-driven reactivation (Babbe et al., 2000; Junker et al., 2007). Similar clonal expansions have not been identified amongst local CD4 + T cell populations (Junker et al., 2007). Furthermore, CD8 + T cells recovered from the cerebrospinal fluid (CSF) of MS patients during disease relapses exhibit enhanced cytotoxic properties when compared to cells obtained during periods of remission (Malmstrom et al., 2008). Finally, many active MS plaques express major histocompatibility complex (MHC) class I antigens on both oligodendrocytes and the axonal processes of neurons making them potential targets of CD8 + T cells (Hoftberger et al., 2004). What remains unclear, however, is the antigenic reactivity of these lesional CD8 + T cells.

Similar to what is seen in MS, activation, homing, and expansion of CD8 + T cells are common host responses following viral infection. If virus-activated CD8 + T cells were to gain entry into the CNS of patients with RRMS, one might predict they could have the potential to initiate events that precipitate a disease relapse. To test this hypothesis, we developed an experimental system in which animals were infected with a normally asymptomatic neurotropic viral pathogen during subclinical demyelinating disease. Specifically, C57BL/6 mice were actively immunized with a peptide of myelin oligodendrocyte glycoprotein (MOG) to induce experimental autoimmune encephalomyelitis (EAE) and then infected with an attenuated strain of Sindbis virus (SV) just prior to EAE onset. These dually diseased animals consistently developed a temporally restricted acceleration of EAE symptoms that depended on virus-induced CD8 + T cells without showing altered expansion or differentiation of myelin-specific CD4 + T cells. Further use of this experimental system will facilitate an improved understanding of how viral infections trigger MS exacerbations and also how CD8 + T cells exert pathogenic effects within active demyelinating lesions.

\* Corresponding author. Tel.: +1 734 615 5635; fax: +1 734 615 7300.  
E-mail address: [davidira@med.umich.edu](mailto:davidira@med.umich.edu) (D.N. Irani).

## 2. Materials and methods

### 2.1. Animal manipulations

Wild type C57BL/6 mice used for most of these experiments were obtained from Harlan Laboratories (South Easton, Massachusetts, USA), while CD8 $\alpha$ -deficient (CD8 $\alpha^{-/-}$ ) mice bred on a C57BL/6 background as well as wild-type controls were purchased from Jackson Laboratories (Bar Harbor, Maine, USA). All animal procedures were performed under isoflurane or avertin anesthesia and were conducted in strict accordance with protocols approved by our University Committee on Use and Care of Animals. Mice were housed under specific pathogen-free, barrier facility conditions on a 10/14-hour light/dark cycle with food and water available ad libitum. The 35–55 amino acid peptide fragment of MOG (MOG<sub>35–55</sub>, amino acid sequence MEVGVYRSPFSRVVHLYRNGK) and the 323–339 amino acid peptide fragment of ovalbumin (OVA<sub>323–339</sub>, amino acid sequence ISQAVHAHAHAEINEAGR) were synthesized and purified by Bio-Synthesis Incorporated (Lewisville, Texas, USA). Active EAE was induced by subcutaneous immunization with 100  $\mu$ g of MOG<sub>35–55</sub> peptide emulsified in Complete Freund's Adjuvant (CFA) containing 4 mg/ml heat-killed *Mycobacterium tuberculosis* H37Ra (Sigma-Aldrich, St. Louis, Missouri, USA) at four sites over the flanks. Sham immunized mice received the equivalent treatment using OVA<sub>323–339</sub> or were given CFA alone. Each immunized mouse also received 300 ng of *Bordetella pertussis* toxin (List Biological Laboratories, Campbell, California, USA) injected intraperitoneally in 0.15 mL of phosphate buffered saline (PBS) on day 0 and day 2 post-immunization. Mice were examined daily for signs of EAE and disease severity was rated in each animal using a standard six-point scale as follows: 0, no discernible deficit; 1, limp tail; 2, impaired gait and/or ability to flip over from a supine position; 3, partial hind-limb paralysis; 4, total hind-limb paralysis; 5, moribund; and 6, dead. Mice were euthanized immediately upon reaching a moribund state.

Wild-type Sindbis virus (SV) strain AR339, biologically cloned from stock SV1A, was grown and assayed for plaque formation in BHK-21 cells as described below. Stock titers of  $1 \times 10^6$  plaque-forming units (PFU)/mL were stored at  $-80^\circ\text{C}$  until use. Virus aliquots were used once and not subjected to repeated freeze-thaw cycles. To induce encephalitis, mice were injected with 1000 PFU of SV suspended in 10  $\mu$ L of PBS via direct intracerebral inoculation into the right cerebral hemisphere. PBS alone was used as a sham inoculation control.

### 2.2. Isolation of tissue-derived mononuclear cells

Anesthetized mice underwent thorough transcardiac perfusion with chilled PBS. Brains and spinal cords were collected, minced into small fragments, and pressed through a 70- $\mu$ m stainless steel mesh sieve into Hank's balanced salt solution (HBSS) containing 10% fetal bovine serum (FBS) before digestion with collagenase (0.2 mg/mL; Worthington Biochemical Corporation, Lakewood, New Jersey, USA) and DNase (28 U/mL; Sigma-Aldrich) for 60 min at  $37^\circ\text{C}$ . Mononuclear cells (MNCs) were then isolated by centrifugation over a 30%/70% Percoll gradient (GE Healthcare Life Sciences, Pittsburgh, Pennsylvania, USA) and washed with HBSS. Splenic and draining lymph node MNCs were collected by homogenizing whole tissues through a fine stainless steel mesh screen and eliminating contaminating red blood cells (RBCs) by hypotonic lysis.

### 2.3. Enumeration of MOG-specific T helper (Th)1 and Th17 cells by ELISPOT assays

Splenic-, lymph node- and spinal cord-derived MNC were harvested from mice on day 11 post-immunization (day 3 post-infection) or day 14 post-immunization (day 6 post-infection) as described above. Pooled cells from a minimum of three animals per group were cultured in triplicate in RPMI 1640 supplemented with 10% FBS, 12.5 mM

HEPES buffer, 1 mM sodium pyruvate,  $1 \times$  non-essential amino acids, 2 mM L-glutamine,  $1 \times$  penicillin–streptomycin, and 50  $\mu$ M 2-mercaptoethanol for 18 h in 96-well filtration plates (EMD Millipore, Billerica, Massachusetts, USA) at densities ranging from 0.5 to  $2.0 \times 10^5$  cells/well (spleen and lymph node) or 0.75 to  $3.0 \times 10^4$  cells/well (spinal cord) with or without the addition of 50  $\mu$ g/mL MOG<sub>35–55</sub> peptide. Cell densities of  $2.5 \times 10^5$  cells/well (spleen and lymph node) or  $3.0 \times 10^4$  cells/well (spinal cord) generally provided optimal cytokine detection. The following antibodies (all from eBiosciences, San Diego, California, USA) were used to detect individual cytokine production by ELISPOT assay: IL-17 (clone TC11-18H10), IL-17-biotin (clone TC11-8H4), IFN- $\gamma$  (clone AN18), IFN- $\gamma$ -biotin (clone R4-6A2), IL-4 (clone 11B11), IL-4-biotin (clone BVD6-24G2), TNF- $\alpha$  (clone 1F3F3D4), TNF- $\alpha$ -biotin (rabbit polyclonal), GM-CSF (clone MP1-22E9), and GM-CSF-biotin (clone MP1-31G6). Streptavidin-alkaline phosphatase (Southern Biotech, Birmingham, Alabama, USA) and an alkaline phosphatase substrate kit (Vector Laboratories, Burlingame, California, USA) were used to identify labeled cells. Spots were counted with a CTL ImmunoSpot Analyzer using ImmunoSpot software (Cellular Technology Limited, Shaker Heights, Ohio, USA). Counts for cells cultured in media alone were subtracted from those for cells stimulated with MOG<sub>35–55</sub> to determine the proportion of antigen-specific T cells having either a Th1 (IFN- $\gamma$  production) or a Th17 (IL-17 production) phenotype. Results presented reflect the numbers of cells collected from a minimum of three animals at each experimental time point normalized to  $1 \times 10^6$  input cells.

### 2.4. Preparation of bone marrow derived dendritic cells (BMDCs) and ex vivo analysis of MOG- and SV-specific T cell cytokine responses

To generate BMDC, femurs and tibiae were removed from C57BL/6 mice and cut at each end. A 10-mL syringe fitted with an 18-gauge needle was used to flush out the bone marrow into sterile RPMI 1640 containing 10% FBS and  $1 \times$  penicillin–streptomycin and then to dissociate cellular aggregates by trituration. Dissociated cells were pelleted by centrifugation at  $300 \times g$  for 5 min at  $4^\circ\text{C}$  and RBCs eliminated by hypotonic lysis. Viable cells were cultured at  $4 \times 10^5$  cells/mL in culture medium supplemented with 20 ng/mL GM-CSF (derived from J558 cell supernatant) at  $37^\circ\text{C}$  for 3 days, at which point the cultures were supplemented with fresh GM-CSF-containing culture medium. On day 6, the culture medium was carefully aspirated, and the loosely adherent BMDCs were gently removed in warmed PBS. A sterile 0.02% EDTA solution also warmed to  $37^\circ\text{C}$  was used to detach the remaining layer of adherent BMDC. Pooled cells were pelleted by centrifugation at  $300 \times g$  for 5 min, and re-cultured at  $4 \times 10^5$  cells/mL in fresh culture medium supplemented with 20 ng/mL GM-CSF at  $37^\circ\text{C}$  for 4 days, and then media were replaced one more time for the last 48 h of culture.

Following 12 days in culture, loosely adherent BMDCs were plated in 6-well plates at  $4 \times 10^5$  cells/mL in culture medium supplemented with 10 ng/mL GM-CSF along with either 50  $\mu$ g/mL MOG<sub>35–55</sub> peptide, SV at a multiplicity of infection (MOI) of 10 or 30 virus particles:target cells, or vehicle alone. After incubating at  $37^\circ\text{C}$  overnight, BMDCs were washed and co-cultured with spinal cord-derived MNC obtained from dually diseased mice at day 14 post-immunization. Cells were mixed at a BMDC: MNC ratio of 1:10 in round-bottomed 96-well culture plates. After 24 h of incubation at  $37^\circ\text{C}$ , culture supernatants were collected and stored at  $-80^\circ\text{C}$  until further use. Samples were later thawed and used at dilutions of 1:5 or 1:10 in ELISA assays for individual T cell-derived cytokines. The following antibodies (all from eBiosciences) were used to measure cytokine levels: IFN- $\gamma$  (clone AN18), IFN- $\gamma$ -biotin (clone R4-6A2), IL-17 (clone TC11-18H10), and IL-17-biotin (clone TC11-8H4). Briefly, 96-well flat-bottomed microtest plates (BD Biosciences) were coated overnight at  $4^\circ\text{C}$  with the capture antibody. After washing with PBS/0.05% Tween 20, the plates were blocked for 1 h at  $37^\circ\text{C}$  with 1% bovine serum albumin (Sigma Aldrich) in PBS. The plates were washed again,

and the culture supernatants and standards (all diluted in the blocking solution) were added to the wells and incubated for 1 h at 37 °C. After more washing, the biotinylated antibody diluted in blocking solution was added to the wells and incubated for 1 h at 37 °C. A horseradish peroxidase-conjugated streptavidin (Southern Biotech) diluted 1:1000 in blocking solution was added to the wells and incubated for 20 min at 37 °C. Plates were washed a final time, and a TMB peroxidase solution (Thermo Scientific, Rockford, Illinois, USA) was applied to the wells to detect antibody binding by developing them at room temperature with the addition of a 10% H<sub>2</sub>PO<sub>4</sub> stop solution when the highest standard appeared saturated. The absorbance at 450 nm in each well was detected using a microplate reader (BioRad, Hercules, California, USA). Results presented reflect the mean  $\pm$  standard error of the mean (SEM) of pg cytokine/mL culture supernatant from triplicate wells.

### 2.5. Virus titration assays

To measure the amount of infectious virus present in CNS tissues, animals were perfused extensively with PBS and brains and spinal cords were extracted, weighed, snap-frozen on dry ice, and stored at  $-80$  °C until virus titration assays were performed. At that time, 20% (w/v) homogenates of each sample were prepared in PBS, and serial 10-fold dilutions of each homogenate were assayed for plaque formation on monolayers of BHK-21 cells. Serial 10-fold dilutions of culture supernatants were similarly assayed for plaque formation on monolayers of BHK-21 cells to measure titers in all viral stocks. Results presented are the mean  $\pm$  SEM of the log<sub>10</sub> of viral plaque-forming units per gram of tissue derived from a minimum of 3 animals at each time point.

### 2.6. Histopathology

On day 14 post-immunization (day 6 post-infection), anesthetized mice were sequentially perfused with PBS and PBS containing 4% paraformaldehyde. Spinal columns were removed, excess was tissue trimmed, and samples were post-fixed overnight in PBS with 4% paraformaldehyde. After rinsing in PBS, spinal columns were decalcified in a formic acid solution overnight and processed for paraffin embedding. Myelin was detected in 4- $\mu$ m thick sections of the lumbar spinal cord by incubating deparaffinized slides overnight at 37 °C in a 0.1% solution of Luxol fast blue suspended in 95% ethanol/0.5% glacial acetic acid. Sections were then differentiated in 0.05% lithium carbonate and counterstained with a 0.1% cresyl violet solution. Viral antigens and virus-infected neurons were detected and quantified using a polyclonal rabbit anti-SV antiserum as previously described (Irani and Prow, 2007). After a final dehydration step, stained sections were coverslipped and imaged using a Nikon Ti-U inverted microscope equipped with a DS-Fi-1 digital camera and supported by the NIS-Elements Basic Research acquisition and analysis software package (Nikon Instruments, Melville, New York, USA). The images shown are representative of at least five stained sections derived from a minimum of three mice per group.

### 2.7. Flow cytometric analysis of CNS-derived inflammatory cell populations

Spinal cord-derived inflammatory cells were resuspended in PBS containing 2% FBS and stained with fluorescently conjugated primary antibodies followed by flow cytometric analysis on a FACSCanto II flow cytometer (BD Biosciences, San Jose, California, USA). To quantify individual cell populations, cell suspensions were stained with antibodies against CD3, CD4, CD8, CD11b, CD19, CD45, Ly-6C, and Ly-6G (all from eBiosciences). A minimum of 30,000 events within a defined forward and side scatter gate containing CD45+ cells were analyzed to determine the proportion of each cell type in each experimental sample. The total number of each cell population present in individual brain or spinal cord specimens was then calculated by multiplying the total number of viable gradient-isolated cells from each sample (counted on a hemacytometer) by the proportion of cells labeled with each

antibody. Results presented reflect the average number of cells per spinal cord in isolates pooled from a minimum of ten animals in each experimental group.

### 2.8. Measurement of CNS tissue inflammatory mediators by Luminex assays

Anesthetized mice were perfused extensively with PBS and individual brains and spinal cords collected, snap-frozen on dry ice, and stored at  $-80$  °C until use. Thawed tissue was later homogenized in 0.5 ml of PBS containing a protease inhibitor cocktail (Complete Mini EDTA-free Protease Inhibitor Cocktail Tablets, Roche Diagnostics, Indianapolis, Indiana, USA) and an RNase inhibitor (RNasin, Promega BioSciences, San Luis Obispo, California, USA). Homogenates were centrifuged to pellet all remaining tissue debris, total protein content was measured in each extract, and diluted supernatants were used for subsequent Luminex analysis. Milliplex mouse CD8+ T cell Magnetic Bead Panels (EMD Millipore, Billerica, Massachusetts, USA) cytokine detection systems were used according to the manufacturer's instructions to quantify cytokine and chemokine levels in brain and spinal tissue extracts. Plates were read on a Luminex 200 instrument (EMD Millipore), and cytokine and chemokine concentrations (pg/ml) were calculated by the BioPlex manager software (BioRad) using standard curves. Results presented reflect the mean  $\pm$  SEM of cytokine or chemokine quantities per milligram of total protein extracted from brain or spinal cord tissue from a minimum of 3 animals at each time point.

### 2.9. Statistical comparisons

The Prism 5.0 software package (GraphPad Software, La Jolla, California, USA) was used for all statistical analyses. A two-way analysis of variance (ANOVA) test was applied when comparing different experimental groups over time. Unpaired Student's *t* tests were used to assess differences between different experimental groups at single time points. In all cases, differences at a *p* < 0.05 level were considered significant.

## 3. Results

### 3.1. Mice infected with SV exhibit an accelerated onset of EAE

To determine whether an otherwise asymptomatic viral infection of the CNS altered the clinical course of EAE, we established a dual disease paradigm where C57BL/6 mice were actively immunized with MOG<sub>35–55</sub>, followed by direct intracerebral inoculation of SV eight days later (EAE + SV group). Control groups were either immunized with MOG<sub>35–55</sub> and then sham-inoculated with PBS (EAE + sham group), immunized with OVA<sub>323–339</sub> in CFA or CFA alone and then infected with SV (sham + SV), or left unimmunized and simply challenged with SV (SV alone). We found that EAE + SV mice consistently developed an accelerated clinical course compared to EAE + sham animals (Fig. 1, top). Both groups had 100% disease incidence and eventually reached the same peak disease scores, after which they maintained similar levels of chronic disability. Neither sham + SV mice (Fig. 1) nor SV alone mice (data not shown) ever developed any discernable neurological deficits. When footpad rather than intracerebral viral challenge following MOG immunization was tested, the timing and severity of EAE symptoms did not differ from a sham inoculated control group (Fig. 1, bottom). These data show that SV-dependent acceleration of EAE symptoms requires virus entry into the CNS, the preferred site of replication *in vivo*. They support prior studies demonstrating that while weanling mice challenged peripherally with SV develop local subcutaneous virus replication and a transient viremia, they do not progress to acute encephalomyelitis (Johnson et al., 1972).



### 3.2. Myelin antigen-specific T cell responses are unaffected by superimposed SV infection

While peripheral inoculation of SV did not affect EAE severity compared to sham-inoculated controls (Fig. 1, bottom), the priming and/or differentiation of encephalitogenic CD4<sup>+</sup> T cells in peripheral lymphoid tissues might be influenced by signals induced by CNS infection. We therefore analyzed MOG-specific T cell cytokine production by ELISPOT assays using spleen-, lymph node- and spinal cord-derived MNC collected on day 14 post-immunization (day 6 post-infection), where the mean clinical disease scores were most divergent between the SV-infected and sham-inoculated groups (Fig. 1, top). These assays showed similar frequencies of MOG-specific CD4<sup>+</sup> lymphocytes producing the pro-inflammatory cytokines, IFN- $\gamma$  and IL-17A, in both the peripheral lymphoid tissues and the CNS of EAE + SV and EAE + sham mice (Fig. 2). Furthermore, the proportions of MOG-specific cells producing other disease-associated cytokines, including IL-4, TNF- $\alpha$ , and GM-CSF, were similarly unaffected by superimposed SV infection, as were cells derived from the spleens and lymph nodes of animals on day 11 post-immunization (day 3 post-infection) corresponding to EAE onset (data not shown). These findings demonstrate that acute SV infection accelerates EAE symptoms via a mechanism independent of the priming and maintenance of autoreactive Th1 and Th17 CD4<sup>+</sup> T cell responses.

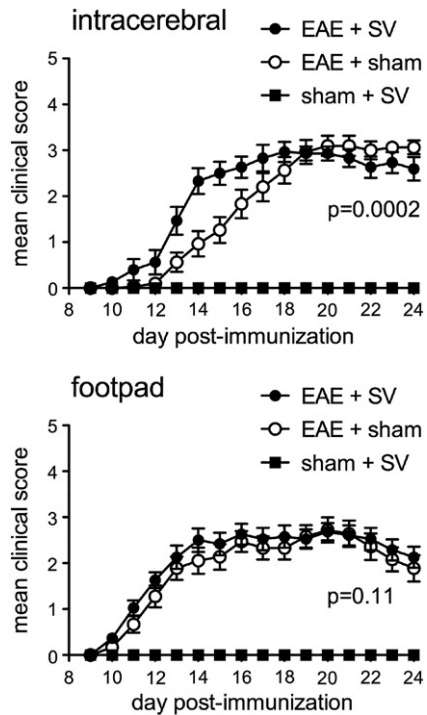
### 3.3. Virus-specific T cells accumulate in the CNS of EAE mice with superimposed SV infection

To investigate virus-specific T cell responses by CNS-derived MNC from EAE + SV mice, we used whole virus particles in an ex

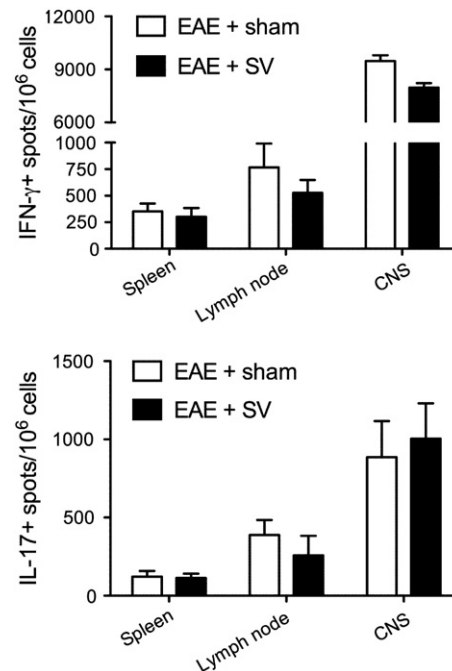
vivo restimulation assay. Naïve bone marrow-derived dendritic cells (BMDCs) were pre-incubated overnight with either MOG peptide or whole SV particles prior to co-culture with MNC isolated from the CNS of EAE + SV mice on day 14 post-immunization. ELISAs were then used to measure IFN- $\gamma$  and IL-17A levels in the co-culture supernatants. MOG-pulsed BMDCs stimulated the most robust cytokine production by CNS-derived T cells (Fig. 3). Virus-pulsed BMDCs elicited significant IFN- $\gamma$  production but no measurable IL-17A levels above baseline, and IFN- $\gamma$  levels increased as more virus particles were used to prime the BMDCs (Fig. 3). Comparable virus-specific cytokine responses were seen with CNS-derived MNC from sham + SV mice, as were MOG-specific responses amongst CNS MNC from EAE + sham mice (data not shown). Neither cell population responded to virus or to MOG when animals had not been infected or immunized, respectively (data not shown). These data show that substantial numbers of both myelin antigen- and virus-specific T cells capable of producing various pro-inflammatory cytokines associated with EAE pathogenesis accumulate in the CNS of our dually diseased mice, and that cross reactivity between myelin and viral antigens does not occur in this experimental system.

### 3.4. Replication and clearance of SV from the CNS is unaffected in mice with underlying EAE

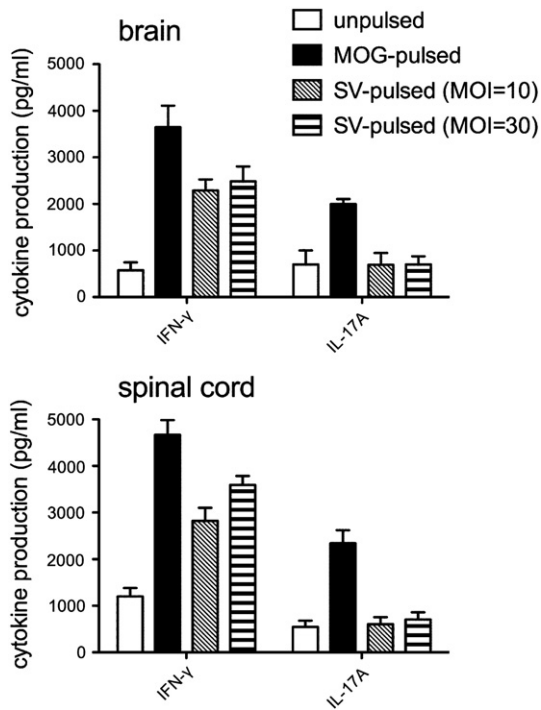
It was previously shown that SV is cleared from the CNS by antibody-dependent mechanisms (Levine et al., 1991), but that IFN- $\gamma$  mediates CNS clearance in a site-specific manner in the absence of B cells (Binder and Griffin, 2001). Based on these findings, pro-inflammatory mediators already present in the CNS of mice with developing EAE might be expected to modulate the replication and/or clearance of SV in a way that could influence the pathogenicity of infection. We therefore compared CNS virus replication curves between sham- and MOG-immunized mice to determine if the enhanced EAE symptoms seen in EAE + SV mice might actually reflect a novel clinical manifestation of the infection itself. Although mean viral titers in the brains of MOG-immunized mice were higher



**Fig. 1.** Clinical disease scores in mice with active immunization EAE and superimposed SV infection. Groups of animals were immunized with either the MOG<sub>35–55</sub> peptide or an irrelevant antigen as described in [Materials and methods](#). Eight days later, just prior to the onset of EAE symptoms, three matched groups then received a direct intracerebral inoculation of either SV or PBS also as described. Three other matched groups received footpad challenges of either SV or PBS. Cohorts of mice ( $n = 15–19$  per group) were scored on a daily basis using an established rating scale. Clinical disease scores (mean  $\pm$  SEM) plotted over time were compared by two-way ANOVA. While intracerebral viral challenge clearly accelerated EAE symptoms (top panel), peripheral virus inoculation had no effect on the subsequent course of EAE (bottom panel). No animal receiving a sham immunization developed any clinical symptoms following SV infection via either route (filled squares).



**Fig. 2.** Comparison of MOG-specific cytokine production by peripheral and CNS-derived T cells isolated from either EAE + SV or EAE + sham mice on day 14 post-immunization. Cells were prepared and ELISPOT assays were performed as described in [Materials and methods](#). The frequency of IFN- $\gamma$ -producing (top panel) and IL-17A-producing (bottom panel) T cells from all three tissues of both cohorts was identical ( $p > 0.05$  for all comparisons by Student's  $t$ -test).

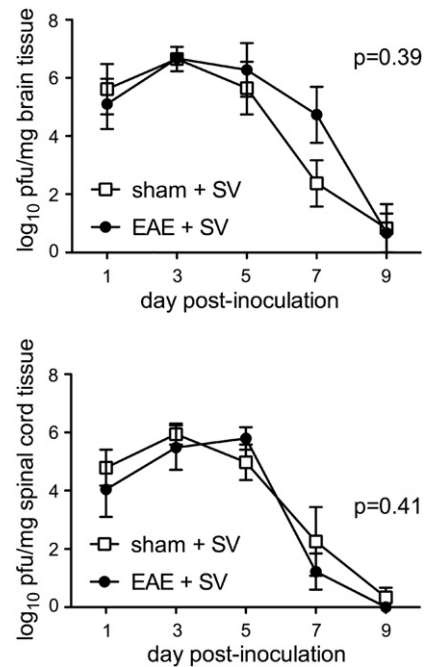


**Fig. 3.** T cells derived from the CNS of dually diseased animals respond to MOG-pulsed and SV-pulsed DCs in vitro. Effector cells were isolated from CNS tissue; bone marrow-derived DCs were isolated, cultured and pulsed with antigen; and co-cultures between effector and stimulator cells were performed as described in *Materials and methods*. While both IFN- $\gamma$  and IL-17A were detected by ELISA in co-culture supernatants when MOG-specific responses were examined, viral antigens elicited only an IFN- $\gamma$  response in cells derived from both the brain (top panel) and spinal cord (bottom panel). DCs pulsed with a higher multiplicity of infection (MOI) of SV evoked somewhat greater IFN- $\gamma$  production. No measurable virus-specific IL-17A response could be detected at either MOI (i.e., virus particle-to-DC ratio) examined.

on day 7 following viral challenge compared to sham-immunized mice with SV infection, the onset and peak of virus replication were similar between the two groups (Fig. 4, top). Moreover, viral titers fell to undetectable levels in both the brains and spinal cords of SV-infected EAE mice by day 9 post-inoculation, indicating that viral clearance mechanisms were not significantly impaired by concurrent EAE (Fig. 4). Immunohistochemical staining for viral antigens in CNS tissue sections did not reveal altered virus tropism or greater numbers of infected neurons in the EAE + SV group at any time point (data not shown). Therefore, the enhanced symptoms observed in our dually diseased mice are due to a virus-dependent effect on EAE pathogenesis and not to enhanced or prolonged CNS viral replication.

### 3.5. Enhanced immune cell infiltration in the dually diseased CNS is marked by an over-representation of CD8 + T cells

Detailed histological characterization of spinal cord tissues from our dually diseased mice showed no overt differences in the extent of myelin loss compared to animals with EAE alone, although more robust inflammatory cell infiltration of CNS tissue was noted when infection occurred together with EAE (Fig. 5A–B). By flow cytometry, T cells, monocytes, and neutrophils were all more abundant in the spinal cords of EAE + SV animals compared to both the sham + SV and the EAE + sham groups on day 14 post-immunization (Fig. 5C). T cell counts in particular differed to the greatest degree between EAE + SV and EAE + sham mice (Fig. 5C). When individual T cell subsets were quantified, more CD3+/CD8+ cells were enumerated in the spinal cords of EAE + SV animals than was expected by the sum of those present as a result of infection or EAE alone (Fig. 6B). An explanation for why the sum of CD4+ and CD8+ T cells in this group of mice is lower than the



**Fig. 4.** Replication and clearance of SV from both the brain (top panel) and spinal cord (bottom panel) is unaffected by the presence of underlying EAE. Cohorts of mice were immunized and infected as described in *Materials and methods*. Triplicate samples were obtained at each time point for each of the two experimental groups, and the amount of infectious virus present in tissue was determined by plaque titration assay as described. Mean  $\pm$  SEM of the  $\log_{10}$  viral titer per mg of tissue is shown; neither set of brain or spinal cord replication curves were different from each other over time by two-way ANOVA.

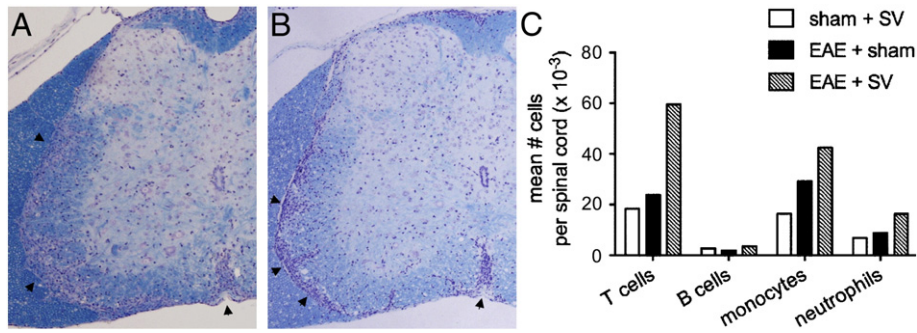
earlier group (Fig. 5C) is likely due to differences in the exact timing of EAE onset between the two cohorts. No differences in the number and proportion of CD8 + T cells isolated from the brains of EAE + SV and EAE + sham mice were found (data not shown). These data suggest that CD8 + T cells are recruited to the spinal cords of dually diseased animals as a result of a synergistic, rather than an additive, effect of the autoimmune and anti-viral immune responses.

### 3.6. SV infection induces the expression of multiple inflammatory factors associated with CD8 + T cell responses in the CNS of mice with underlying EAE

Tissue homogenates from EAE + sham and EAE + SV mice were analyzed using a multiplex, bead-based assay to quantify the expression of factors known to either derive from or to influence CD8 + T cells. Samples obtained from animals 1, 3, and 6 days after either sham or virus inoculations were examined for expression trends over time. Results showed that all levels of CD137, granzyme B, IFN- $\gamma$ , MIP-1 $\beta$  and RANTES were disproportionately increased in spinal cords derived from dually diseased animals (Fig. 7). There were no differences between the two experimental groups with respect to levels of these factors expressed in the brain (Fig. 7). Collectively, these findings suggest a scenario where SV infection superimposed on EAE increases the recruitment of CD8 + T cells expressing high levels of cytolytic mediators directly into target tissues. This recruitment and mediator release, in turn, triggers a cascade of changes that transiently accelerates EAE pathogenesis and clinical disease symptoms.

### 3.7. Deletion of CD8 + T cells prevents SV-induced worsening of clinical EAE symptoms

In order to directly assess the role of CD8 + T cells in the accelerated EAE seen in the EAE + SV mice, we repeated the clinical assessments of EAE with or without superimposed SV infection using CD8 $\alpha$ -deficient

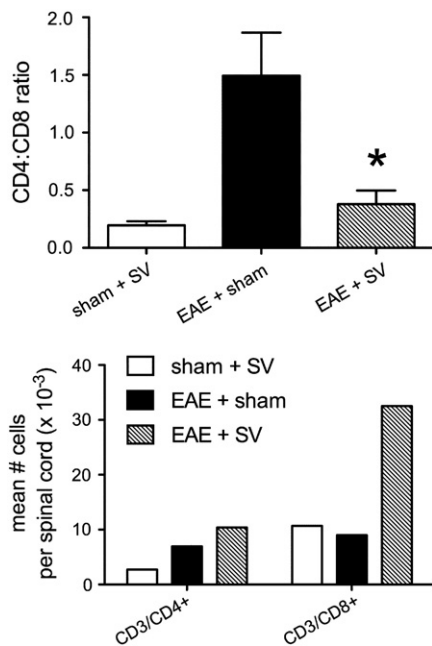


**Fig. 5.** Demyelination and inflammatory cell infiltration in the spinal cords of mice with EAE and superimposed SV infection. Luxol fast blue staining of the lumbar spinal cord from an EAE + sham mouse shows robust mononuclear cell infiltration of white matter (arrowheads), but no significant myelin loss on day 14 post-immunization (A). A corresponding sample from an EAE + SV animal shows somewhat more obvious inflammatory cell infiltration (arrowheads) but no overt evidence of demyelination either (B). Flow cytometry-based quantification of individual inflammatory cell populations in the three experimental groups on day 14 post-immunization shows greater cell numbers in the EAE + SV group, particularly amongst infiltrating T cells (C).

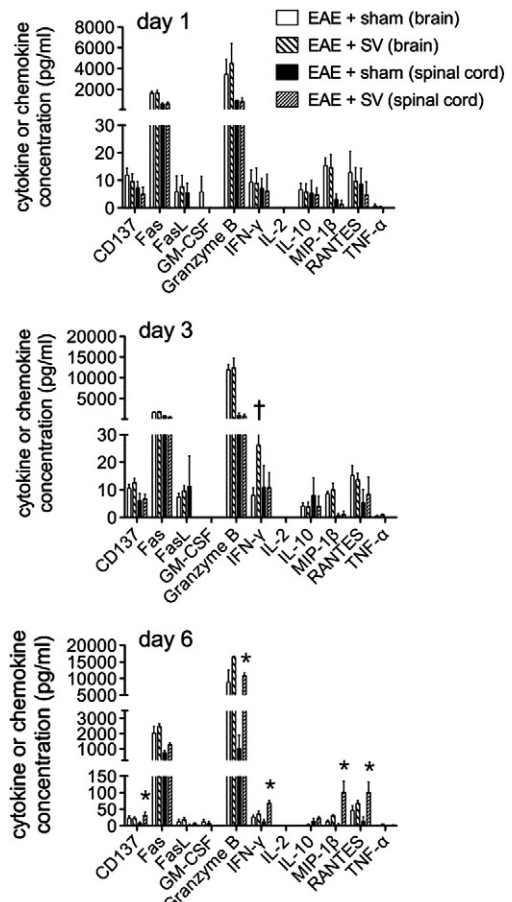
(CD8 $\alpha^{-/-}$ ) mice bred to a C57BL/6 background. We found that virus inoculation of CD8 $\alpha^{-/-}$  mice on day 8 post-immunization did not alter the onset, severity, or subsequent clinical course of EAE when compared to actively immunized CD8 $\alpha^{-/-}$  counterparts given a sham inoculation (Fig. 8). This confirms that while CD8 + T cells are not required for the development of EAE, they do accelerate EAE pathogenesis in the presence of an acute CNS viral infection. Since neither sham + SV nor SV alone mice develop any overt neurological symptoms (Fig. 1 and data not shown), virus-induced CD8 + T cells appear completely innocuous in the setting of infection alone. Our dual disease paradigm provides a model to understand how an acute viral infection can promote transient exacerbations of neurological symptoms in patients with RRMS.

#### 4. Discussion

Abundant epidemiological data suggest that a preceding viral infection can trigger clinical exacerbations in patients with RRMS, but a mechanistic understanding of how this occurs remains elusive (Sibley et al., 1985; Andersen et al., 1993; De Keyser et al., 1998; Edwards et al.,

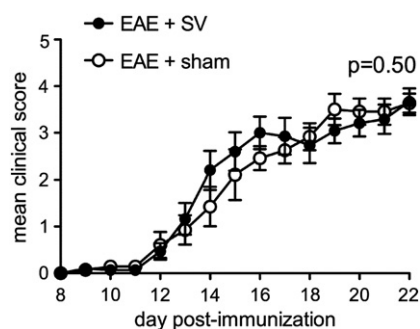


**Fig. 6.** Flow cytometric analysis of the T cell subsets present in the spinal cords of sham + SV, EAE + sham, and EAE + SV mice on day 14 post-immunization. Spinal cord-derived mononuclear cells were pooled from a minimum of 10 mice in each experimental group, stained, and analyzed by flow cytometry as described in Materials and methods. The mean  $\pm$  SEM of the calculated CD4:CD8 ratio from groups of mice in 4 separate experiments shows a significant reversal between EAE + sham and EAE + SV groups ( $*p < 0.05$  by Student's *t*-test) (A). The mean number of CD3+/CD4+ and CD3+/CD8+ T cells per spinal cord was calculated when cells were pooled from a minimum of 10 mice in each experimental group (B).



**Fig. 7.** CD8 + T cell-associated mediators were measured in the brains and spinal cords of EAE + sham and EAE + SV mice using a multiplex bead-based assay system. A minimum of three samples from each experimental group was assayed on day 1 (top panel), day 3 (middle panel) and day 6 (bottom panel) following virus inoculation (day 9, day 11 and day 14 post-immunization, respectively). The only notable difference in the brain samples was higher IFN- $\gamma$  production in EAE + SV mice on day 3 ( $^{\dagger}p < 0.05$  by Student's *t*-test). In the spinal cord, a range of mediators were increased in the EAE + SV group compared to the EAE + sham animals on day 6 post-inoculation ( $*p < 0.05$  for all comparisons by Student's *t*-test).





**Fig. 8.** Clinical disease scores in  $CD8\alpha^{-/-}$  mice with active immunization EAE and superimposed SV infection of the CNS. Groups of  $CD8\alpha^{-/-}$  animals were immunized with the MOG<sub>35–55</sub> peptide as described in *Materials and methods*. Eight days later, just prior to the onset of EAE symptoms, each group then received a direct intracerebral inoculation of either SV or PBS also as described. Cohorts of mice (EAE + SV, EAE + sham;  $n = 8$  each) were then scored on a daily basis using an established clinical rating scale. Clinical disease scores (mean  $\pm$  S.D.) plotted over time were compared by two-way ANOVA.

1998; Kriesel et al., 2004; Correale et al., 2006). Even the clues from population-based studies showing that infections causing fever or infections lasting longer than five days are particularly likely to cause MS flares have failed to yield substantial mechanistic insight into this phenomenon (Sibley et al., 1985; Andersen et al., 1993; De Keyser et al., 1998). The fact that current disease-modifying therapies only partially reduce the frequency of MS attacks reinforces the importance of elucidating how environmental triggers such as viral infections predispose to disease relapses; such findings could lead to advances in patient management. Here, we find that an asymptomatic alphavirus infection of the CNS accelerates EAE onset in a temporally restricted manner through a mechanism driven by virus-induced  $CD8 + T$  cells – cells that do not ordinarily cause any CNS tissue damage in infected animals without underlying EAE. We find no evidence that the autoreactive  $CD4 + T$  cell response is in any way perturbed by the superimposed infection. These data raise interesting questions as to the pathogenic properties of  $CD8 + T$  cells within demyelinating lesions, and our dual disease paradigm provides a novel experimental system to investigate how these cells can worsen disease in a transient manner.

A handful of prior studies have reported that preceding viral infections (either CNS or systemic in nature) predispose rodents to more frequent or severe EAE, although none offer a mechanistic explanation for this phenomenon (Massanari et al., 1979; Mokhtarian and Swoveland, 1987; Liebert and ter Meulen, 1993; Mokhtarian et al., 1994; Peacock et al., 2003; Verbeek et al., 2007). Most recently, mice with a latent gammaherpesvirus infection were reported to develop more severe EAE, and although this virus did not actually reach the CNS, that virus-specific  $CD8 + T$  cells infiltrated both the brains and spinal cords of these dually diseased animals (Casiraghi et al., 2012). This study also found that the  $CD4 + T$  cell response within the CNS of mice at peak EAE was highly skewed towards a Th1 profile when animals had a latent gammaherpesvirus infection (Casiraghi et al., 2012). While intriguing, neither of these findings could be causally linked to the accelerated disease symptoms. To our knowledge, only three previous publications have reported on the effects of superimposed viral infection during established EAE; two studies showed that the spread of Semliki Forest virus infection to the CNS hastened EAE symptoms (Soilu-Hanninen et al., 1997; Jerusalem et al., 2003), while a third report demonstrated that intracerebral injection of herpes simplex virus type-1 during EAE remission precipitated a relapse in a proportion of recipients (Hochberg et al., 1977). Again, none of these studies addressed the cellular and molecular pathways through which the clinical deterioration occurred. A detailed mechanistic understanding of such events could be critical to developing therapies that inhibit virus-induced MS relapses.

An important difference between human MS and rodent EAE relates to the composition of the cellular infiltrates within and around demyelinated lesions. While  $CD4 + T$  cells drive EAE in most models, MS lesions often show a preponderance of  $CD8 + T$  cells (Liblau et al., 2002; Neumann et al., 2002; Junker et al., 2007). As to a role for  $CD8 + T$  cells in EAE pathogenesis, Najaflan et al. showed that  $CD8$ -deficient mice developed more severe EAE than wild type controls, and that the adoptive transfer of  $CD8 + /CD28 - T$  cells into  $CD8$ -deficient hosts caused significant disease attenuation (Najaflan et al., 2003). Since then,  $CD8 + /CD122 +$  regulatory T cells (Treg) that dampen EAE have been described (Lee et al., 2008), and these Treg acting via TGF- $\beta$  and IFN- $\gamma$  have been characterized in some detail (Chen et al., 2009). Several groups have reported that MOG-specific  $CD8 + T$  cells actually kill MOG-loaded  $CD4 + T$  cells in a perforin-dependent manner to suppress EAE (Beeston et al., 2010; York et al., 2010). Conversely, the minimal MOG epitope, MOG<sub>37–46</sub>, was shown to be recognized by  $CD8 + T$  cells and to be capable of inducing EAE via active immunization (Ford and Evavold, 2005). A MOG<sub>37–50</sub>/H-2D<sup>b</sup> MHC tetramer could then be used to identify MOG-specific  $CD8 + T$  cells in the CNS both prior to and after EAE onset (Ford and Evavold, 2005), and two groups showed that MOG-specific  $CD8 + T$  cells were actually encephalitogenic in adoptive transfer models (Huseby et al., 2001; Sun et al., 2001). In one of these reports, transferred  $CD8 + T$  cells were incapable of inducing disease in beta-2 microglobulin-deficient hosts, but they persisted over a long-term in susceptible recipients (Sun et al., 2001). Thus, substantial controversy persists related to the role of  $CD8 + T$  cells in EAE, and little is known about their regulatory or pathogenic potential during MS in humans. In our study, we observed more severe EAE in  $CD8\alpha^{-/-}$  mice, but the fact that superimposed SV infection did not further worsen EAE in these hosts suggests that  $CD8 + T$  cells recruited to the CNS by infection are themselves pathogenic in the setting of dual disease (Fig. 8). Our dual disease model could be used to address the complex molecular mechanisms of  $CD8 + T$  cells in inflammatory demyelinating diseases compounded with a CNS viral infection.

In the Theiler's virus-induced demyelinating disease model, Myoung et al. recently found that  $CD8 + T$  cells specific for an epitope present in the viral capsid protein, VP3, played an unexpected causative role in initiating the chronic demyelinating phase of disease (Myoung et al., 2012). These disease-causing, virus-specific  $CD8 + T$  cells had high avidity for infected target cells and produced significant amounts of the inflammatory cytokine, IL-17 (Myoung et al., 2012). In the same experimental system, others have found that CNS-infiltrating  $CD8 + T$  cells come into direct contact and form immunological synapses with infected neurons (McDole et al., 2010), while antiviral  $CD8 + T$  cells isolated from infected mice and then cloned in vitro can transfer an EAE-like disease when injected into naive recipients (Libbey et al., 2012). Detailed characterization of these encephalitogenic  $CD8 + T$  cells derived from infected hosts showed that they express dual T cell receptors as a potential explanation for their viral- and self-reactivity (Libbey et al., 2012). These data show that in a model of CNS viral infection by itself, virus-induced  $CD8 + T$  cells can be induced to cause demyelination and overt neurological deficits. Along with our findings, they advocate for more careful consideration of the role played by these cells at the intersection of viral infection and demyelinating disease, at least in the setting where the pathogen actively resides within the CNS.

Unanswered questions arising from our studies primarily relate to whether  $CD8 + T$  cells directly accelerate neurological deficits in mice with EAE, or whether they instead act in a way that transiently potentiates another disease pathway. During SV encephalomyelitis induced in mice without underlying EAE,  $CD8 + T$  cells neither participate in viral clearance from the CNS nor induce any neuropathological damage (Hirsch and Griffin, 1979; Levine et al., 1991). These cells nonetheless are recruited to the CNS in substantial numbers over the course of acute infection (Moench and Griffin, 1984), even if their antigenic specificity and local function remain poorly understood. Our data show that CNS-derived T cells from dually diseased animals make significant

amounts of IFN- $\gamma$  in response to viral antigens (Fig. 3), suggesting that their transient augmentation of EAE symptoms could be occurring in an antigen-specific manner. Attempts to augment symptoms in dually diseased mice via adoptive transfer of virus-specific CD8<sup>+</sup> T cells have been limited by the lack of a tetramer reagent to specifically identify and purify this cell population. Nonetheless, the success of such an approach could eventually allow us to more definitely prove that SV-induced acceleration of clinical EAE is due to an antiviral CD8<sup>+</sup> T cell response. We believe it is unlikely that these cells are reacting directly to myelin antigens, as tissue demyelination is not accelerated in these hosts (Fig. 5). Ongoing studies aim not only to understand how CD8<sup>+</sup> T cells drive this disease-exacerbating effect, but also to determine whether the effect is attributable to specific subpopulations of these cells.

In conclusion, we have generated an experimental system whereby mice with subclinical EAE develop a transient acceleration of disease symptoms via infection with an attenuated neurotropic alphavirus. The infection appears to leave autoreactive CD4<sup>+</sup> T cells unperturbed, and instead worsens disease via the actions of virus-induced CD8<sup>+</sup> T cells that are ordinarily innocuous in infected mice without underlying EAE. We argue that this paradigm has important clinical relevance, as it suggests a mechanism for how viral infections might trigger MS exacerbations in humans and also provides a unique platform to study how CD8<sup>+</sup> T cells might exert pathogenic effects within demyelinating lesions. Further use of this model could lead to the development of interventions that block at least one type of environmentally driven MS exacerbation.

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