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In Vivo Depletion of CD8$^+$ T Cells Prevents Lesions of Demyelination in Semliki Forest Virus Infection

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Following intraperitoneal infection of BALB/c mice with the A7(74) strain of Semliki Forest virus, the virus spreads to the central nervous system (CNS) and initiates an acute inflammatory reaction which includes lesions of primary demyelination. This demyelination is dependent upon activated T lymphocytes. To determine whether CD4$^+$ or CD8$^+$ T cells are involved in the pathogenesis of the demyelination, we have investigated the course of infection in animals treated with monoclonal anti-CD4 or anti-CD8 antibodies. In the normal course of infection, virus was detectable in the brain by infectivity assay and in situ hybridization for up to 14 days. Antiviral immunoglobulin M (IgM) and all subclasses of IgG were produced. From day 10 to 21 postinfection lesions of inflammatory demyelination were present, most notably in the cerebellum and corpus callosum but also in other white matter tracts. Administration of anti-CD4 antibodies removed CD4$^+$ cells from the spleen, prevented production of antiviral IgG, increased virus titers in the brain, and increased demyelination. Administration of anti-CD8 antibodies depleted CD8$^+$ cells from the spleen and did not affect antiviral IgG synthesis or spread of brain virus but reduced CNS inflammatory responses and virtually abolished lesions of demyelination. Administration of both antibodies depleted both T-cell subsets from the spleen, prevented IgG antibody production, increased brain virus, and abrogated both CNS inflammation and lesions of demyelination. In conclusion, the CNS demyelination induced by Semliki Forest virus can be prevented by in vivo depletion of CD8$^+$ T lymphocytes.

Following intraperitoneal infection of weanling mice of most strains, the A7(74) strain of Semliki Forest virus (SFV) replicates in the muscles, producing a plasma viremia. Virus then crosses cerebral endothelial cells to infect the central nervous system (CNS) (8, 17, 19, 21). Within the CNS, virus infection is restricted in neurons and the infection remains localized to small perivascular foci (8). If the virus crosses cerebral endothelial cells in the vicinity of a white matter tract, glial cells can become infected (8, 16). Virus can be detected in the brain from 1 to 7 days postinfection by infectivity assay on BHK cells and until day 10 by in situ hybridization (8). A predominantly mononuclear cell inflammatory response is first detectable at day 4 by histological studies, and a pleocytosis is present from day 5 to 9 (13, 14). The inflammatory response lasts for several weeks. The blood-brain barrier is leaky from day 4 to 10, at the time of maximum virus titers, and antiviral immunoglobulin G (IgG) is synthesized within the CNS after this time (15). Lesions of inflammatory, primary demyelination are present scattered throughout the CNS. These are first visible under the light microscope at 10 days postinfection, immediately after the virus has been cleared. Demyelination continues until 3 or 4 weeks postinfection and is followed by remyelination (12, 16). During infection, changes in axonal transport in optic nerves have been reported, and the animals have abnormal visually evoked responses (11, 25, 26). The system provides a good model of virus-induced demyelination and multiple sclerosis (7).

Immunologically, termination of the transient plasma viremia which is apparent from 1 to 3 days postinfection coincides with increasing titers of antiviral IgM. Later, antiviral IgG antibodies of all subclasses are produced (8). Antiviral cytotoxic T cells are present from 5 to 17 days postinfection (2). Immunosuppression, using total body irradiation, antithymocyte serum, cyclophosphamide, or cycloleucine, can delay or prevent the lesions of demyelination (1, 10, 24). In athymic *nu/nu* mice, the virus establishes a persistent infection with no CNS damage and no lesions of demyelination. Lesions can be restored by reconstitution of the *nu/nu* mice with T cells and are induced sooner and are more severe if sensitized T cells are transferred (6, 9).

To determine whether CD4$^+$ or CD8$^+$ T cells mediate the lesions of demyelination, we have depleted these T-cell subpopulations in vivo by using monoclonal antibodies and have studied the course of the infection. The study consisted of four experimental groups, each containing 24 3- to 4-week-old female BALB/c mice (Cambridge University, Department of Pathology, Animal Unit, or Olac Laboratories, Bicester, Oxon, United Kingdom). One group was treated with anti-CD4 antibody, another was treated with anti-CD8, the third received both anti-CD4 and anti-CD8, and the fourth received phosphate-buffered saline (PBS). The antibody inoculum was 1 mg of monoclonal rat anti-CD4 (YTS 191) (5) or 1 mg of monoclonal rat anti-CD8 (YTS 169) (5) and was given intravenously in 0.1 ml of PBS, 2 days before and 2 days after infection. The antibody preparations were partially purified Igs derived from pooled ascitic fluids by ammonium sulfate precipitation. Mice receiving anti-CD4 antibody had additional intraperitoneal injections at 4-day intervals. To avoid anaphylactic shock, no further doses of anti-CD8 were given. All mice were inoculated intraperitoneally with 5,000 PFU of SFV A7(74) in 0.1 ml of PBS at day 0. Three mice were sampled from each group at days 4, 7, 10, 12, 14, 21, 28, and 35 postinfection. Spleens were analyzed by fluorescence-activated cell sorter (FACS), and the numbers of CD4$^+$, CD8$^+$, and CD3$^+$ cells were determined. CNS virus infection was determined by virus plaque assay and in situ hybridization as detailed previously (8). The titers of antiviral antibodies in the sera were determined by enzyme-linked immunosorbent assay (ELISA) (8). Inflammatory and demyelinating changes in the

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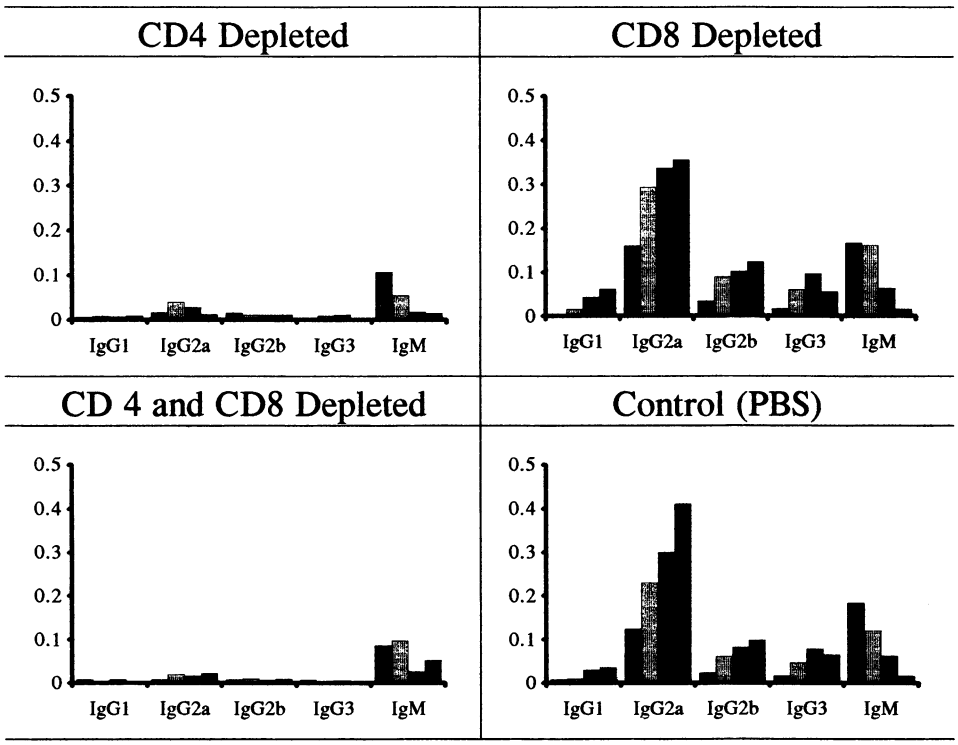

FIG. 1. IgM and IgG subclass antiviral responses to SFV infection. Each bar shows the mean for two mice. For each subclass, titers (optical density at 570 nm) are shown from left to right at 7, 10, 14, and 21 days postinfection. The sera at a dilution of 1/1,000 were tested by ELISA using IgM and IgG subclass-specific antibodies (8).

CNS were determined by microscopy. All mice remained healthy for the course of the study except for those in the group depleted of both $CD4^+$ and $CD8^+$ T cells; mice in this group appeared thin, hunched, and unwell by day 10 and continued to look unwell throughout the study. Mice in this group were sampled up to and including day 21, during which time none of the mice in the group died; however, the three mice remaining after day 21 died before the day 28 sampling point.

At days 4, 7, 10, 14, and 21, spleens were removed from two mice in each group and from two control, age-matched uninfected mice. Splenocytes positive for the phenotypic markers CD3, CD4, and CD8 were enumerated by FACS analysis. Within each group at each sampling point and at different times postinfection, there was some variability between mice in the relative numbers of lymphocytes positive for these three markers. The gated lymphocyte population of control uninfected animals typically had 20% $CD4^+$ and 8% $CD8^+$ cells. Values were more variable for infected animals, with 15 to 30% CD4 and 6 to 15% CD8 cells, depending upon the animal and the time postinfection. In animals treated with anti-CD4 or anti-CD8, the percent $CD4^+$ or $CD8^+$ cells, respectively, was consistently less than 1% and usually as low as 0.2%.

Figure 1 shows the IgM and IgG subclass antiviral responses in the four groups of mice. Intact animals and animals depleted of $CD8^+$ cells produced equivalent titers of antiviral IgM and IgG of all subclasses. Depletion of $CD4^+$ cells or $CD4^+$ and $CD8^+$ cells abolished the IgG response, indicating that the CD4 depletion regimen was effective at a functional level. The IgM response remained intact in all groups.

The presence of virus in the brain was determined both by plaque assay on BHK cells and by in situ hybridization. Infectivity titers for the four groups (Fig. 2) were essentially similar, although titers on days 4 and 7 were higher in the brains of mice in the CD4-depleted and doubly depleted groups than in those in the control or CD8-depleted groups. Infectious virus could not be detected in any of the groups after

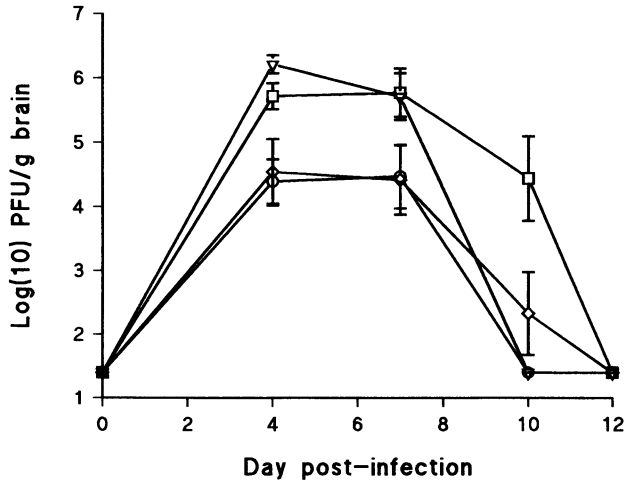

FIG. 2. Virus titers in the brains of the four groups of mice, CD4 depleted (□), CD8 depleted (◇), CD4 and CD8-depleted (▽), and control (○). Each point represents the mean value for three mice; error bars indicate the standard errors of the mean.

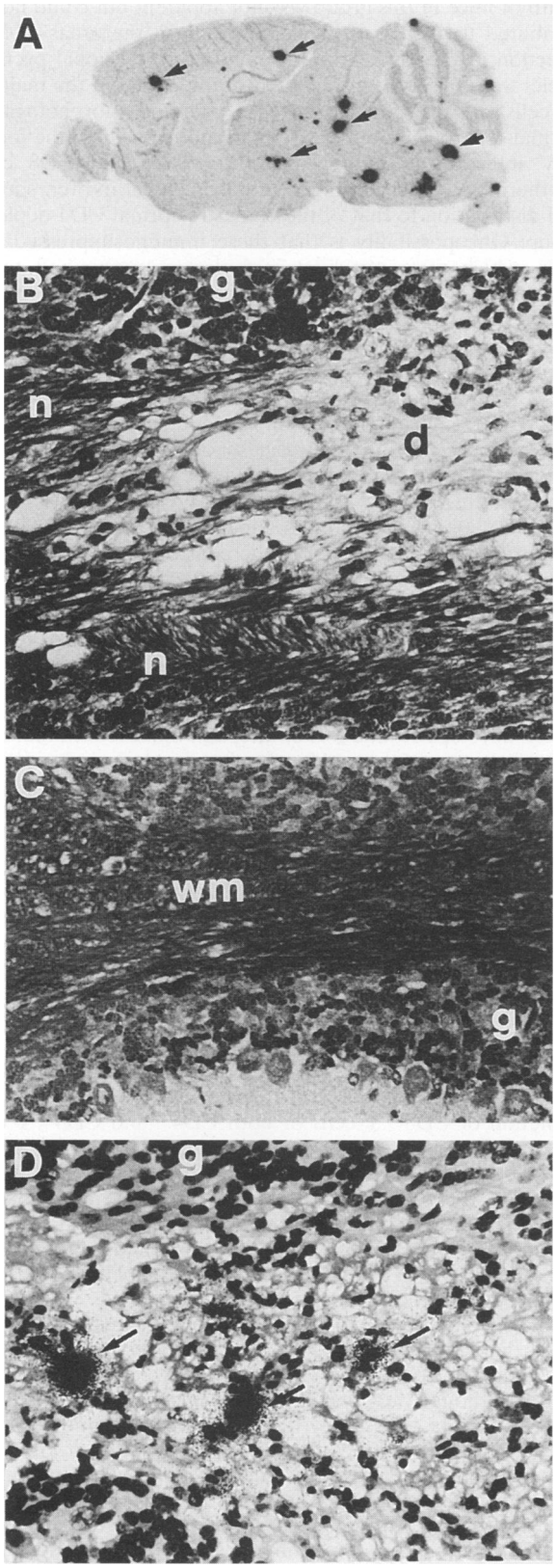

FIG. 3. (A) Autoradiographic analysis of a sagittal section from a control mouse 7 days after infection, processed by in situ hybridization. The black foci (arrows) contain cells positive for viral RNA. (B) Inflammation and demyelination (d) in a white matter tract of the cerebellum. The lesion contains many mononuclear inflammatory cells. Intact normal myelin (n) can be seen adjacent to the lesion in this section from a CD4-depleted animal 14 days postinfection stained with luxol fast blue and cresyl violet. (C) White matter (wm) tract from the cerebellum of a CD8-depleted animal 14 days postinfection, with neither mononuclear inflammatory cells nor demyelination. The section was stained with luxol fast blue and cresyl violet. (D) Inflammatory demyelination in the cerebellum of a CD4-depleted animal 14 days postinfection. The section was processed for in situ hybridization to show cells positive for viral RNA (arrows) and was counterstained with hematoxylin and eosin. In panels B, C, and D, the granule cell layer of the cerebellum (g) is visible.

TABLE 1. Number of mouse brains in which viral RNA was detectable by in situ analysis at various days postinfection (p.i.)[a]

Day p.i.	No. of brains with virus			
	CD4 depleted	CD8 depleted	CD4 and CD8 depleted	Control
4	2	1	2	1
7	3	3	3	3
10	2	1	1	1
14	1	0	2	0
21	0	0	0	0

[a] For each group at each time point, three sections were examined from each of three brains (see Fig. 3).

day 10. An example of the in situ hybridization analysis and the number of brains positive by this assay at each time point in each group are shown in Fig. 3 and Table 1, respectively. In all four groups, between days 4 and 14 small perivascular foci of infection were observed scattered throughout the brain. At any given time point, the number of these foci present in each brain varied from section to section. Three sections were examined for each brain. There was no apparent difference in the numbers or sizes of foci in the brains of mice from the four different groups. Foci were most numerous at day 7 when the infectivity titers were highest. No viral RNA could be observed by in situ hybridization after day 14. The similar infectivity titers, distributions of viral RNA, and time courses of virus clearance in all four groups indicate that neither $CD4^+$ T cells, $CD8^+$ T cells, nor IgG antibodies are required for clearance of infectious virus from the brain. The higher infectivity titers at days 4 and 7 in mice depleted of $CD4^+$ cells demonstrates that although not required to control brain virus, these cells do have some effect on titers, most likely by providing help for production of neutralizing IgG antibodies. Previous studies have indicated that antiviral IgM alone is sufficient to clear the viremia (8, 9). In all four experimental groups, antiviral IgM synthesis was unaffected; either this antibody alone is also sufficient to control infectious virus in the brain or the CNS infection is self-limiting. The possibility that virus persists after day 10 at levels below that detectable by BHK cell plaque assay cannot be ruled out. Indeed, we had suspected that virus would persist in the CD4- and CD8-depleted animals, since we have previously (6, 9) detected persistent low levels of SFV A7(74) in athymic *nu/nu* mice by 50% intracerebral lethal dose titration in 0- to 2-day-old mice. However, this detection method is approximately 100-fold more sensitive than the BHK cell plaque assay used in the present study (unpublished observation). A persistent infection similar to that seen previously in *nu/nu* mice is unlikely to have been detected. By in situ hybridization analysis of three random sections from each brain, viral RNA was detectable in two of three doubly depleted animals on day 14 compared with zero of three in

TABLE 2. Inflammation (I) and demyelination (D) in brains of mice

Day p.i.[a]	Mean score for group[b]							
	CD4 depleted		CD8 depleted		CD4 and CD8 depleted		Control	
	I	D	I	D	I	D	I	D
4	1	0	0	0	0	0	1	0
7	3	0	2	0	0	0	2	0
10	8	5	4	0	1	0	11	3
14	8	7	5	0	0	0	11	5
21	10	5	4	2	0	0	3	2
28	1	1	0	0	NA[c]	NA	1	0
35	0	0	0	0	NA	NA	0	0
Total	31	18	15	2	1	0	29	10

[a] p.i., postinfection.
[b] Scores were determined as described in the text.
[c] NA, not applicable, since all mice in this group had died by day 28.

both the CD8-depleted and control groups, suggesting delayed clearance; however, no virus was detectable in any mice by this method at day 21.

Hematoxylin- and eosin-stained and luxol fast blue- and cresyl violet-stained sagittal sections from five different levels of each mouse brain were examined microscopically and scored on a scale of 1 to 5 for inflammation and demyelination. All slides were coded and examined without bias as to their origin. The scores for the three mice in each group at each time point were added and divided by the number of sections examined (15), to give a mean score which is shown in Table 2. Examination of the brains of mice in the CD4-depleted, CD8-depleted, and control (PBS) groups demonstrated an inflammatory response from day 7 to 21. This consisted of scattered, usually perivascular foci of infiltrating mononuclear cells. At 7 and 12 days, many of the inflammatory foci were observed by in situ hybridization to contain cells positive for viral RNA. At these time points, foci of inflammation without virus-positive cells and foci of infection without inflammation were rare. After 14 days, inflammatory foci remained but virus positive cells were no longer observed. Foci containing cells positive for viral RNA and inflammatory mononuclear cells were observed in both grey and white matter. Following intraperitoneal infection, SFV infects the CNS, producing small scattered, often perivascular foci of infection (8). The inflammatory response during SFV encephalitis has also been described as scattered and perivascular (12, 13). From the present study, it is clear for the first time that the scattered, inflammatory foci correspond to the scattered foci of infection.

In contrast to the other three groups, with the exception of one of the three mice on day 10, which had a small lesion of mononuclear cell inflammation, the animals depleted of both CD4 and CD8 T cells had no mononuclear cell inflammatory response in the brain at days 4, 7, 10, 12, 14, and 21. However, all three animals sampled on day 14 had a florid meningitis consisting mostly of neutrophils and the brains of these animals contained many debris-laden macrophages. This neutrophil meningitis was also present in some animals in this group, though reduced in severity, on days 10 and 21. No meningitis was observed in our previous studies of *nu/nu* mice (6, 9), and unlike the *nu/nu* mice which developed a clinically inapparent, lifelong persistent CNS infection, all the mice in the CD4 and CD8-depleted group were unwell after day 10, and the three mice remaining in the study after day 21 unexpectedly died by day 28. The cause of sickness and late death of mice in this group was not apparent but could not be attributed to increased neuropathology in the areas of SFV infection in the CNS. In all four groups, occasional pycnotic nuclei were observed in the areas of infection but the majority of cells positive for viral RNA appeared morphologically normal. There was no increase in pycnotic nuclei in the foci of SFV infection in mice depleted of CD4 and CD8 cells. Furthermore, the CNS infection was identical in titer, spread, and distribution to that of the clinically normal CD4-depleted group. One possibility is that these immunosuppressed animals, which were not kept in isolators, succumbed to an opportunistic infection of which neutrophil meningitis was a sign.

Lesions of inflammatory demyelination were observed in luxol fast blue- and cresyl violet-stained sections on days 10, 14, and 21 in both control mice and mice depleted of $CD4^+$ cells (Fig. 3B). Demyelination was most frequent in the cerebellum and corpus callosum but was also observed in other white matter tracts. At days 10 and 14, some of the inflammatory, demyelinating lesions contained cells positive for viral RNA (Fig. 3D). Demyelination was enhanced in the CD4-depleted mice relative to that shown in the control mice, possibly as a result of more widespread CNS infection, as indicated by the higher titers of virus in the brain. Lesions of demyelination were always inflammatory. At 10 and 14 days, occasional cells in these lesions were positive for viral RNA. This is the first report demonstrating the presence of virus-infected cells in the lesions of demyelination, and the results suggest that demyelination develops around foci of infection that occur in the white matter. In contrast to this, despite a strong inflammatory response, demyelination was absent in brains of mice depleted of $CD8^+$ cells (Fig. 3C) with the exception of one animal with lesions of demyelination on day 21. None of the doubly depleted mice had lesions of demyelination.

Clearly, demyelination does not result from direct viral destruction of cells, since in the absence of an inflammatory response these lesions do not develop. This is consistent with our previous findings for athymic *nu/nu* mice, which, despite persistence of virus in the brain, do not develop demyelination (6, 9). Our previous adoptive transfer studies have shown demyelination to be dependent upon T cells (9). In the present study, depletion of $CD4^+$ T cells and abolition of IgG antibody synthesis did not prevent but enhanced demyelination, whereas depletion of $CD8^+$ T cells or both $CD4^+$ and $CD8^+$ T cells prevented demyelination. The single exception to this was one CD8-depleted mouse at day 21 which did have demyelinating lesions. This could have resulted from the return of some $CD8^+$ T cells. In these experiments, the animals were not thymectomized; depletion of $CD8^+$ cells could therefore only be expected to be transient, with new cells being continually recruited from the bone marrow.

That depletion of $CD8^+$ cells prevents demyelination suggests that demyelination results from direct $CD8^+$ T-cell-mediated destruction of virally infected cells in the white matter, although the possibility that demyelination is mediated by toxic factors released by these $CD8^+$ cells cannot be ruled out. The nature of the target cell(s) is not clear from the present study, but previous studies by ourselves and others have shown that the A7(74) strain of SFV can infect oligodendrocytes (8, 16). An important question is why these cells are not destroyed by the infection itself. The A7(74) infection is restricted in neurons and oligodendrocytes of 3- to 4-week-old mice, and viral proteins are produced, but electron microscopy studies indicate that no or few infectious virus particles are assembled. This restricted replication results in minimal cell

destruction and prevents viral spread, resulting in the observed small, scattered perivascular foci of infection (8, 18, 20).

It appears most likely that cytotoxic antiviral T cells are directly responsible for the demyelination. Infected oligodendrocytes can be targets for cytotoxic T lymphocytes, since viral proteins are produced during the restricted infection and oligodendrocytes are inducible for class I major histocompatibility complex molecules (4). Temporal development of demyelination is also consistent with $CD8^+$ T-cell-mediated destruction of virally infected cells. Lesions are first apparent at 10 days, at which time virus-infected cells are present in the early lesions and virus-specific cytotoxic T cells can be detected in the spleen (2). That demyelination was most extensive at 14 days, 4 days after the virus was no longer detectable by infectivity assay, probably reflects the relative insensitivity of the plaque assay and the time taken for macrophages to remove the myelin debris and reveal the full extent of lesions.

The role of $CD8^+$ cells has also been investigated in Theiler's virus infection: results are controversial but it appears that $CD8^+$ cells protect against the spread of infection and in vivo depletion results in increased demyelination (3, 22, 23, 27). The present study is the first case in which depletion of $CD8^+$ T cells clearly prevents lesions of demyelination following a CNS virus infection. We suggest that lesions of inflammatory CNS demyelination observed following SFV infection result from $CD8^+$ T-cell-mediated destruction of cells, presumably oligodendrocytes, in which viral replication is restricted and not directly cytolytic.

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