Targeting Inflammatory Demyelinating Lesions to Sites of Wallerian Degeneration

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In Theiler’s murine encephalomyelitis virus (TMEV) infection, an animal model for multiple sclerosis (MS), axonal injury precedes inflammatory demyelinating lesions, and the distribution of axonal damage present during the early phase of infection corresponds to regions where subsequent demyelination occurs during the chronic phase. We hypothesized that axonal damage recruits inflammatory cells to sites of Wallerian degeneration, leading to demyelination. Three weeks after TMEV infection, axonal degeneration was induced in the posterior funiculus of mice by injecting the toxic lectin *Ricinus communis* agglutinin (RCA) I into the sciatic nerve. Neurpathology was examined 1 week after lectin injection. Control mice, infected with TMEV but receiving no RCA I, had inflammatory demyelinating lesions in the anterior/lateral funiculi. Other control mice that received RCA I alone did not develop inflammatory lesions. In contrast, RCA I injection into TMEV-infected mice induced lesions in the posterior funiculus in addition to the anterior/lateral funiculi. We found no differences in lymphoproliferative responses or antibody titers against TMEV among the groups. This suggests that axonal degeneration contributes to the recruitment of inflammatory cells into the central nervous system by altering the local microenvironment. In this scenario, lesions develop from the axon (inside) to the myelin (outside) (Inside-Out model). (Am J Pathol 2007, 171:1563–1575; DOI: 10.2353/ajpath.2007.070147)

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS). Although its etiology is unknown, epidemiological data and clinical reports support the association of MS with environmental factors, particularly viral infection. In recent years, pathological and neuroimaging studies have re-emphasized the importance of axonal damage in MS. Immunostaining studies have provided direct evidence for axonal damage using antibodies against two markers of injured axons: nonphosphorylated neurofilament protein (NFP) and amyloid precursor protein.

Several groups have demonstrated that axonal damage occurred early during the course of MS and that damaged axons were found not only in active demyelinating lesions but also in periplaque white matter without demyelination, remyelinating lesions and normal-appearing white matter. Magnetic resonance spectroscopy analyses have also demonstrated gray matter lesions and axonal injury in normal-appearing white matter in MS patients. Axonal damage in normal-appearing white matter has also been found in an animal model for MS, coronavirus-induced demyelination. Although axonal damage in MS is believed to occur secondarily after the destruction of the myelin sheath or oligodendrocytes, the myelin-forming cells, these findings suggest that axonal damage could be independent of demyelinating activity.

Wallerian degeneration is the anterograde degeneration of axons caused by injury to the proximal part of the axon or by death of the neuronal cell body. In most cases, the demonstration of Wallerian degeneration in humans has been limited to compact neuronal tracts, such as the corticospinal tract and optic radiation. In MS, Wallerian degeneration was believed to be rare; this was probably attributable to a difficulty in detection because MS lesions are usually not confined to a single pathway or tract. However, more recently, magnetic resonance imaging, magnetic resonance spectroscopy, and autopsy studies have documented the presence of Wallerian degeneration in some patients with MS.
Theiler's murine encephalomyelitis virus (TMEV) infection of mice has been used as an experimental model to study viral and immune-mediated pathogenic mechanisms in MS.\textsuperscript{1,20,21} TMEV belongs to the family Picornaviridae. Intracerebral infection with TMEV in susceptible mice causes an inflammatory demyelinating disease with virus persistence in macrophages and glial cells $\sim 1$ month after infection. Demyelination is accompanied by T-cell and macrophage infiltration of the anterior and lateral funiculi of the spinal cord, but the posterior funiculus and the brain are generally spared.\textsuperscript{22–24} In TMEV infection, ultrastructural studies demonstrated that degenerating axons are found in all states of myelination—completely demyelinated, remyelinated, or normally myelinated—thus suggesting Wallerian degeneration after axonal transection occurring in lesions located elsewhere in the CNS.\textsuperscript{25} Previously, we demonstrated that axonal degeneration precedes inflammatory demyelination, and the distribution of damaged axons observed during the early phase corresponded to regions where subsequent demyelination occurs during the chronic phase.\textsuperscript{24} This indicates that axonal degeneration is not a simple consequence of demyelination but rather a potential trigger for recruitment of T cells and macrophages into the CNS leading to subsequent loss of myelin.

Here, we hypothesized that axonal injury recruits inflammatory cells into sites of Wallerian degeneration leading to demyelination. We used an approach for induction of Wallerian degeneration in the CNS, which involves injecting \textit{Ricinus communis} agglutinin (RCA) I (a toxic lectin) into the peripheral nervous system (PNS). RCA I injected into the sciatic nerve is transported axonally (retrograde) leading to death of dorsal root ganglion cells and Wallerian degeneration of the posterior funiculus in the spinal cord. Three weeks after TMEV infection, we injected RCA I into the sciatic nerve of SJL/J mice. Neuropathologically, mice that received TMEV but no RCA I had demyelinating lesions only in the anterior and lateral funiculi. In contrast, RCA I injection in TMEV-infected mice induced lesions not only in the anterior and lateral funiculi but also in the posterior funiculus. This suggests that axonal degeneration itself contributes to recruitment of inflammatory cells into the CNS, targeting lesion development. Here, lesions develop from the axon (inside) to the myelin (outside) (Inside-Out model).\textsuperscript{12,26}

\textbf{Materials and Methods}

\textit{Animal Experiments}

A working stock of the Daniels (DA) strain of TMEV, prepared in baby hamster kidney (BHK)-21 cells (American Type Culture Collection, Manassas, VA), was used

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{\textbf{a}: RCA I was injected into the sciatic nerve using a microinjector. \textbf{b}: The RCA I solution was labeled with Fast Green FCF to ensure the intraneural injection. \textbf{c}: RCA I injected into the left sciatic nerve is transported axonally, leading to death of dorsal root ganglion (DRG) cells and axonal degeneration of the left half of the posterior funiculus, ipsilateral to the injection site. We hypothesized that this axonal degeneration would recruit inflammatory cells into the posterior funiculus.}
\end{figure}
for all experiments. Six-week-old female SJL/J mice (Jackson Laboratory, Bar Harbor, ME) were injected with 2 × 10^6 plaque-forming units (PFUs) of DA virus intracerebrally or 1 × 10^6 PFU of DA virus intravenously. Three weeks after injection, the left sciatic nerve was exposed surgically under anesthesia. We injected 2 μg of RCA I (Vector Laboratories, Burlingame, CA) in a 0.4-μl solution into the left sciatic nerve using a manual microinjector (Sutter Instrument Company, Novato, CA) fitted with a 500-μl syringe (Figure 1a). The RCA I solution contained 0.1% Fast Green FCF (Fisher Scientific, Pittsburgh, PA) to monitor the intraneural injection (Figure 1b). The skin was closed with wound clips. Control mice were injected with phosphate-buffered saline (PBS) in the presence or absence of Fast Green FCF, or received no surgery. Fast Green FCF is a synthetic organic dye and widely used in food and scientific research. After surgery, mice were placed on the Deltaphase isothermal pad (Braintree Scientific, Inc., Braintree, MA) to keep the body temperature a constant 37°C. One week after surgery, mice were euthanized with isoflurane (IsoSol; Vedco Inc., St. Joseph, MO). Animal handling and protocols were approved by the Institutional Animal Care and Use Committee at the University of Utah.

**Histology**

Mice were euthanized and perfused with PBS followed by a 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) solution. CNS tissues were removed, and the surface of the injected side was labeled by India ink (Kuretake, Nara, Japan). The brains were divided into five coronal slabs and the spinal cords into 12 transverse slabs. The tissues were embedded in paraffin. Four-μm-thick sections were stained with Luxol fast blue for myelin visualization. Histological scoring was performed as described previously. For scoring spinal cord sections, each spinal cord section was divided into quadrants: the anterior funiculus, the posterior funiculus, and each lateral funiculus. Any quadrant containing meningitis, perivascular cuffing, or demyelination was given a score of 1 in that pathological class. The total number of positive quadrants for each pathological class was determined, divided by the total number of quadrants present on the slide, and multiplied by 100 to give the percent involvement for each pathological class. An overall pathology score was also determined by assigning a positive score if any pathology was present in the quadrant. This was also presented as percent involvement. Brain sections were scored for meningitis (0, no meningitis; 1, mild cellular infiltrates; 2, moderate cellular infiltrates; and 3, severe cellular infiltrates), perivascular cuffing (0, no cuffing; 1, 1 to 10 lesions; 2, 11 to 20 lesions; 3, 21 to 30 lesions; 4, 31 to 40 lesions; and 5, more than 40 lesions), and demyelination (0, no demyelination; 1, mild demyelination; 2, moderate demyelination; and 3, severe demyelination). Each score from the brain was combined for a maximum score of 11 per mouse.

**Lymphoproliferative Assay**

Before performing the assays, antigen-presenting cells (APCs) infected with DA virus (DA-APCs) or sham infected (n-APCs) were made from whole spleen cells isolated from naive SJL/J mice using 0.83% NH4Cl in Tris-HCl buffer. The spleen cells were infected in vitro with DA virus at a multiplicity of infection of 1 or sham-infected, incubated overnight, and irradiated with 200 rads using a 137Cs irradiator.

We harvested and pooled spleens from TMEV-infected mice and isolated mononuclear cells (MNCs) with Histopaque-1083 (Sigma-Aldrich). Cells, suspended in a volume of 200 μl containing 2 × 10^5 cells in RPMI 1640 medium supplemented with 1% glutamine, 1% antibiotics, 50 μmol/L 2-mercaptoethanol, and 10% fetal bovine serum, were added to each well of 96-well plates. Cells were stimulated with i) live DA virus at a multiplicity of infection of 1; ii) 1 μg of purified DA virus antigen inactivated by UV irradiation; iii) 2 × 10^5 cells of DA-APCs; or iv) n-APCs or modified myelin proteolipid protein (PLP)_{139–151} peptide (HSLGKWLGHPDKF) (final concentration of peptide, 50 μg/ml; Peptide Core Facility, University of Utah Huntsman Cancer Institute, Salt Lake City, UT). The cells were cultured at 37°C in 5% CO2 for 4 days and then pulsed with 1 μCi of [methyl-3H]thymidine (PerkinElmer Life and Analytical Sciences, Boston, MA) per well and cultured for another 18 to 24 hours. Cultures were harvested onto filters using a Combi cell harvester (Skatron Instrument AS, Lier, Norway), and 3H incorporation was measured using an LS 6500 multipurpose scintillation counter (Beckman Coulter, Inc., Fullerton, CA).

**Enzyme-Linked Immunosorbent Assay**

At the time of sacrifice, 1 month after DA virus infection, sera were collected and stored at −20°C. Titers of TMEV-specific antibodies were determined by enzyme-linked immunosorbent assay as described previously. TMEV antigen was prepared by infecting 80 to 90% confluent monolayers of BHK-21 cells with DA virus at a multiplicity of infection of 0.1 PFU/cell in Dulbecco’s modified Eagle’s medium containing 2% fetal bovine serum, using tissue culture dishes (150 × 20 mm; Sarstedt Inc., Newton, NC). When most of the cells showed cytopathic effects (18 hours after infection), media were removed, and cells were washed three times in ice-cold PBS, scraped, and resuspended at 4 × 10^7 cells/ml in PBS.
The cells were subjected to three freeze-thaw cycles. After the final thaw, the cells were sonicated with a microtip probe sonicator (Sonifier 250; Branson Ultrasonics Corporation, Danbury, CT) on ice. The cellular solution was centrifuged at 1500 rpm for 10 minutes at 15°C, and the supernatant was saved. The supernatant was then centrifuged at 100,000 × g for 1 hour. The pellet was resuspended in PBS and sonicated as before. The protein concentration was determined using the Bio-Rad protein assay (Bio-Rad Laboratories Inc., Richmond, CA). TMEV antigen solution was stored at −70°C with 0.5 mmol/L thimerosal (Sigma-Aldrich). Ninety-six well plates were coated overnight with TMEV antigen at 4°C. After blocking with diluent [PBS containing 10% bovine calf serum (Cosmic Calf Serum; HyClone, Logan, UT) and 0.2% Tween 20], twofold dilutions of the mouse sera beginning at 1:27 were added to the plates and incubated at room temperature for 90 minutes. After washing with PBS containing 0.2% Tween 20, the plates were incubated with a peroxidase-labeled goat anti-mouse immunoglobulin (Ig) G (H + L) (Life Technologies, Gaithersburg, MD) in diluent for 90 minutes. The plates were colorized with o-phenylenediamine dihydrochloride (Sigma-Aldrich) and read at 492 nm on a Titertek Multiskan Plus MK II spectrophotometer (Flow Laboratories, McLean, VA). The endpoint of the assay was determined as the reciprocal of the highest dilution that gave an optical density reading that was 2 SDs more than the control baseline of sera from uninfected mice.

Results

Induction of CNS Axonal Damage by Toxin Injection in the PNS

We used a technique, previously established by Yamamoto and colleagues,33–35 to induce Wallerian degeneration in the CNS by way of retrograde axoplasmic transport of RCA I from the PNS without disturbing the CNS integrity. By this route, exogenous substances can reach the CNS from the periphery circumventing the blood-brain barrier (BBB). Because RCA I can be transported via axonal...
flow, injection of RCA I into the sciatic nerve leads to the death of dorsal ganglion cells and subsequent degeneration of the ascending sensory tract that localizes in the posterior funiculus. We injected RCA I into the left sciatic nerve of naïve mice to determine whether it could induce axonal degeneration in the left half of the posterior funiculus, corresponding with the side of the injected sciatic nerve (Figure 1c). One week after injection, the spinal cord appeared normal and neither demyelination nor inflammation was detected by Luxol fast blue staining (Figure 2c). Axonal swelling was noted in several nerve fibers by immunohistochemistry against nonphosphorylated NFP (Figure 2a). No microglial activation or T-cell infiltration was seen by lecin histochemistry or immunohistochemistry against CD3 (Figure 2d), respectively. Damaged axons were not observed in the spinal cords of PBS-injected mice or naïve mice (Figure 2b).

**Targeting Inflammatory Demyelinating Lesions to Sites of Axonal Damage in Mice Infected with TMEV**

Three weeks after intracerebral infection, mice were divided into three groups and received i) no treatment (no Tx), ii) PBS injection, or iii) RCA I injection into the left sciatic nerve. One week later, CNS pathology was compared among the groups. During this early chronic phase of TMEV infection, 4 weeks after infection, mice developed MNC infiltration in the meninges and perivascular spaces with demyelination in the white matter of the spinal cord, but not in the brain. In all three groups of mice, the anterior funiculus and the ventral root exit zone (VREZ) were most severely involved (Figure 3, a and b). As we reported previously, we found few lesions in the posterior funiculus of control TMEV-infected mice receiving no treatment (Figure 3d). In contrast, in TMEV-infected mice receiving RCA I, the posterior funiculi were demyelinated in several segments (Figure 3c). The demyelinating lesions were usually seen only in the left side (ipsilateral to the injection side) of the posterior funiculus (Figure 3c). In the demyelinated area, we detected microglia/macrophage and T-cell infiltration (Figure 3, e and g). Control TMEV-infected mice receiving no treatment had infiltrating cells comprised of macrophages and T cells in the anterior and lateral funiculi, but not in the posterior funiculus (Figure 3, f and h). Interestingly, we found changes in the posterior funiculus in some segments in the TMEV-infected control group receiving PBS. We tested whether there was a significant increase in spinal cord pathology by comparing spinal cord pathology scores between the groups.

We quantified the spinal cord pathology and found that the TMEV-infected mice receiving RCA I had significantly more lesions in the left side than the right side of the posterior funiculus; and comparing the left side, the TMEV-infected mice receiving RCA I had significantly more lesions than the no treatment group ($P < 0.01$, Figure 4a). Although TMEV-infected mice receiving PBS tended to have more lesions in the posterior funiculus of the spinal cord compared with the no treatment group, there was no statistical difference in the right side or the left side of the posterior funiculus between the PBS versus no treatment groups ($P = 0.5$, analysis of variance). There was also no significant difference in pathology scores between the right versus left sides of the posterior funiculus in the PBS-injected group ($P > 0.05$).

In the brain, no demyelinating lesions were detected. Only a mild MNC infiltration in the meninges and perivascular areas in the midbrain and brainstem was present. There was no difference in the lesion distribution or brain pathology scores between the groups (mean brain pathology score ± SEM: no Tx, 1.3 ± 0.3; PBS, 1.6 ± 0.3; RCA I, 1.9 ± 0.4; $P > 0.05$, analysis of variance).

**Distribution of Viral Antigen-Positive Cells**

Using immunohistochemistry, we compared the distribution of viral antigen-positive cells in the spinal cord between the groups. In all groups, viral antigen-positive cells were detected only in the white matter of the spinal cord. These infected cells appeared to be glial cells and/or macrophages, which would be morphologically consistent with what we and others have previously reported (Figure 5, e–g). Similarly, in all groups, we detected similar numbers of viral antigen-positive cells in the anterior and lateral funiculi, including VREZ (Figure 5, c and d). In the posterior funiculus, however, substantial numbers of viral antigen-positive cells were seen only in infected mice injected with RCA I (Figure 5a), whereas a few viral antigen-positive cells were detected in infected mice receiving PBS or infected mice without treatment (Figure 5b). In infected mice receiving RCA I, viral antigen-positive cells were usually seen only in the left half of the posterior funiculus, ipsilateral to the injection side.

We also quantified the numbers of viral antigen-positive cells in the CNS. During the chronic phase of TMEV infection, only a few viral antigen-positive cells were seen despite the presence of severe inflammatory demyelinating lesions. In all groups of mice, we found a small number of viral antigen-positive cells per spinal cord segment. There were no significant differences in the total numbers of viral antigen-positive cells among the groups (mean number of viral antigen-positive cells per spinal cord segment ± SEM: no Tx, 3.7 ± 1.2; PBS, 2.4 ± 0.7; RCA I, 3.9 ± 1.2; $P > 0.05$, analysis of variance). In all groups, we detected viral antigen-positive cells mostly in the anterior funiculus and VREZ (Figure 4b). In the posterior funiculus, few viral antigen-positive cells were detected in the no injection group or PBS-injected group (no statistical difference between the two groups, $P > 0.05$). In infected mice receiving RCA I, we found an increase in the number of viral antigen-positive cells in the injected side (left side) of the posterior funiculus; the number was statistically higher than that of the noninjection side and the PBS-injected group ($P < 0.01$, analysis of variance). In the posterior funiculus of the RCA I-injected group, the number of viral antigen-positive cells in the injection side (left) were higher than that of the noninjection side (right), although the numbers did not show a statistical difference (right, 0.04 ± 0.04; left, 0.22 ± 0.06; $P = 0.07$).
Figure 3. Spinal cord pathology of TMEV-infected mice with (a, c, e, and g) or without (b, d, f, and h) RCA I injection. a and c: TMEV-infected mice receiving RCA I injection in the left sciatic nerve had lesions not only in the anterior and lateral funiculi (arrow) but also in the left half (L) of the posterior funiculus (arrowhead). The left side of the spinal cord was labeled by India ink (double arrows). Inflammatory demyelination was seen in the left half of the posterior funiculus, the ipsilateral side of the RCA I injection, but not in the right half (R). b and d: TMEV infection alone induced inflammatory demyelinating lesions in the anterior funiculus and the VREZ (arrow), but not in the posterior funiculus. In the posterior funiculus, macrophage (e and f) and T-cell (g and h) infiltration was seen in RCA I-treated mice (e and g), but not in control mice injected with TMEV alone (f and h). a–d: Luxol fast blue stain. e and f: Lectin histochemistry. g and h: Immunohistochemistry against CD3. Magnifications: ×26 (a and b); ×61 (c–h).
cells in inflammatory demyelinating lesions. This was unexpected because we hypothesized that axonal damage alone could contribute to the recruitment of inflammatory cells in the left posterior funiculus, ipsilateral to the RCA I injection site, without an increase in virus. Thus, we tested whether the number of virus antigen-positive cells in the left posterior funiculus of infected mice receiving RCA I correlated with perivascular cuffing or demyelination. We found that virus persistence did not correlate with perivascular cuffing ($r = 0.59, P = 0.16$) or demyelination ($r = 0.35, P = 0.44$) in the left posterior funiculus of the RCA I-injected group (Figure 6, a and b). This suggests that there was no association between virus persistence and neuropathology in the left posterior funiculus, which is in accord with our prediction. Thus, virus persistence did not play a major role in induction of neuropathology at the posterior funiculus ipsilateral to the RCA I injection site. The presence of virus antigen-positive cells in this area could be attributable to recruitment of virus-infected macrophages.

We next determined whether virus persistence correlated with neuropathology in the other areas of the spinal cord of the RCA I-treated group and control groups. We found significant correlations between virus persistence and perivascular cuffing in some areas (for example, left VREZ in the PBS group; $r = 0.83, P < 0.05$; Figure 6c), whereas other areas showed no significant correlation (data not shown). In most areas in all three groups, virus persistence strongly correlated with perivascular cuffing versus demyelination. Virus persistence did not correlate with demyelination (eg, left VREZ in the PBS group; $r = 0.62, P = 0.1$; Figure 6d).

**Lymphoproliferative Responses against TMEV**

Virus-specific T-cell responses can play either a pathogenic role in demyelination or a protective role through virus clearance in TMEV infection. Because RCA I injection or the injection procedure itself could influence immune reactivity, we compared cellular immune responses against TMEV between infected mice without treatment and mice with PBS or RCA I injection. Spleen MNCs were harvested and incubated without antigen or with UV-irradiated purified DA strain of TMEV (DA Ag), live DA virus, or DA-APCs. Because TMEV-infected mice have been shown to develop autoreactive immune responses in the spleen, MNCs were also stimulated with irradiated uninfected syngeneic spleen cells (n-APC). All MNCs from the three groups of treated mice showed similar lymphoproliferative responses against not only TMEV but also n-APC (autoproliferation) (Figure 7a). No differences were found among the groups. Lymphoproliferative responses against PLP (139–151) were low and showed no significant difference among the three groups (stimulation index: no Tx, 2.3 ± 0.6; PBS, 2.2 ± 0.4; RCA I, 2.9 ± 0.8). Because a low level of anti-PLP lymphoproliferative response is seen even in naïve mice, stimulation indexes of more than 3 were regarded as significant in this system.
Humoral immune responses against TMEV have also been shown to play an important role in TMEV pathogenesis. To exclude the possibility that differences in antibody responses against TMEV accounted for differences in the lesion distribution between the different treatment groups, we titrated serum anti-TMEV antibodies by enzyme-linked immunosorbent assay, when mice were sacrificed 1 week after treatment and 4 weeks after TMEV infection. We detected high anti-TMEV antibody titers in all groups (Figure 7b). There was no statistical difference in antiviral antibody titers.
between TMEV-infected mice without treatment (no Tx), with PBS injection, or with RCA I injection (P > 0.05, analysis of variance). Thus, alteration of the lesion distribution in RCA I-injected mice could not be explained by alteration of anti-TMEV antibody responses.

**Figure 6.** Correlation of virus persistence with inflammation or demyelination in the spinal cord of infected mice. We compared numbers of virus antigen-positive cells with pathology scores of perivascular cuffing (a and c) or demyelination (b and d) of infected mice, 1 week after RCA I or PBS injection. a and b: In RCA I-injected mice, there was no significant correlation of virus persistence with inflammation (a) or demyelination (b) in the left posterior funiculus, ipsilateral to RCA I injection site. c and d: In the left VREZ of PBS-injected mice, virus antigen-positive cells correlated with perivascular cuffing (c) but not with demyelination (d).

**Figure 7.** Cellular (a) and humeral (b) immune responses against TMEV. Three weeks after TMEV infection, groups of mice were untreated (open column, no Tx), sham-injected (hatched column, PBS), or injected with RCA I (closed column, RCA I) into the sciatic nerve. One week after treatment, spleen MNCs and sera were collected. a: Lymphoproliferative responses of MNCs that were incubated without antigen (no Ag) or with purified UV-irradiated antigen from Daniels strain of TMEV (DA Ag), live DA virus (live DA), antigen-presenting cells infected with DA virus (DA-APCs), or uninfected syngeneic antigen-presenting cells (n-APCs) at a ratio of 20:1. All MNCs from the three groups had lymphoproliferative responses against not only DA virus but also n-APCs (autoproliferation). No differences were found between the groups. The results are means ± SEM of three experiments. b: Anti-TMEV antibody measured by enzyme-linked immunosorbent assay. No significant difference in anti-TMEV antibody titer was detected between the groups (P > 0.05). Values are mean anti-TMEV antibody titers ± SEM for seven or eight mice per group. Results are representative of three independent experiments.

### Intraneural Injection Did Not Recruit Inflammatory Cells into the CNS of Mice Infected Intravenously with TMEV

In contrast to intracerebral infection of TMEV, infection of TMEV from the periphery causes CNS disease only in a small percentage of infected mice. This could be attributable to a lack of signaling in the CNS that is required for homing of inflammatory cells into the CNS. We investigated whether changes in the microenvironment within the CNS induced by RCA I injection were enough to recruit inflammatory cells into the CNS in mice infected with TMEV from the periphery. We infected mice intravenously with TMEV. Four weeks after infection, mice were divided into three groups: i) no injection, ii) PBS injection, and iii) RCA I injection into the sciatic nerve. One week after injection, we compared neuropathology among the groups. We found only one mouse in the PBS-injected group developed a mild demyelinating disease in the anterior and lateral funiculi, but not in the posterior funiculus of the spinal cord (data not shown). No lesions were found in TMEV-infected mice with RCA I injection or mice with TMEV infection alone.

### Discussion

The primary target in MS is believed to be either myelin itself or the myelin-forming cells, the oligodendrocytes. Although axonal degeneration has been demonstrated in MS and its respective autoimmune model, experimental autoimmune encephalomyelitis (EAE), it is regarded as a secondary event to myelin damage. In this scenario, the lesion develops from the myelin (outside) to the axon (inside) (Outside-In model). In this Outside-In model, axonal degeneration can be regarded as a simple end result. On the other hand, in TMEV infection, we previously found that axonal damage precedes the demyelinating disease and that the distribution of damaged axons observed during the early phase corresponded to regions where subsequent demyelination occurs during the chronic phase of disease (Table 1, pathomechanism 1). Here, lesions develop from the axon (inside) to the myelin (outside) (Inside-Out model).

In the present study, we demonstrated that experimentally induced axonal damage contributes to the recruitment of T cells and macrophages to sites of Wallerian degeneration in mice infected intracerebrally with TMEV. Although we found increased numbers of viral antigen-positive cells at the sites, there was no significant correlation between virus persistence with inflammation or demyelination. Our results suggest that, in TMEV infection, axonal damage is not a simple consequence of demyelination but can target inflammatory demyelinating lesions to sites of Wallerian degeneration (Table 1, pathomechanism 2). Our findings are similar to what has been reported in a rat adoptive-transfer experimental autoimmune encephalomyelitis (EAE) model, in which axonal damage was induced in rats before encephalitogenic cells were adoptively transferred. In this model, besides the typical EAE lesions randomly distributed in...
areas known to be common in rat EAE (mainly in the spinal cord), an unusual distribution of inflammatory lesions was seen in the region where Wallerian degeneration was experimentally induced, ie, in the ipsilateral thalamus, which has a dense reciprocal connection to the cryolesioned neocortex, and in the optic tract and the superior colliculus, after optic nerve severing without disruption of the BBB. In some patients with MS, lesion development has been observed along the corticospinal tract in a pattern consistent with Wallerian degeneration.14–17

The Inside-Out model and Outside-In model are not mutually exclusive and may act synergistically (Table 1, pathomechanism 3).12,26,41 The lesion may develop first from the inside to the outside (eg, neurotropic virus infects and damages neuronal cell bodies and axons, leading to secondary demyelination in the optic tract). However, at the last step, damaged myelin would be phagocytosed by activated macrophages/microglia, followed by myelin antigen presentation in the CNS. This could induce myelin-specific autoimmune responses, which can attack myelin from the outside, and inflammatory demyelination would lead to axonal damage secondarily. In this instance, pathology develops from the outside to the inside. Here, severe axonal damage leads to Wallerian degeneration of the distal stump of axons and trigger the second cascade reaction. Therefore, Inside-Out and Outside-In models can form a vicious cycle, targeting demyelination in our model also depended on the route of infection, because intracerebral infection, but not intravenous infection, resulted in recruitment of inflammatory cells in the CNS. RCA I injection in mice with intravenous infection of TMEV was not sufficient for induction of the lesions in the posterior funiculus of the spinal cord. Differences in the extent of anti-viral immune responses between intracerebral versus intravenous infection may not explain a lack of CNS lesions in mice infected intravenously with TMEV. Intravenous injection of TMEV has been shown to induce active humoral and cellular immune responses against virus,30 but TMEV does not efficiently infect the CNS by this route and dose. Indeed, in our current experiments, we found similar levels of anti-TMEV antibody titers between intracerebral versus intravenous TMEV-infected groups (data not shown).

Table 1. Pathomechanisms in the Inside-Out Model for MS

| Pathomechanism | Animal model | MS |
|----------------|--------------|----|
| 1. Axonal degeneration precedes demyelination | TMEV infection in susceptible mice | Axonal damage in NAWM |
| 2. Axonal degeneration recruits inflammatory cells | Coronavirus infection | Wallerian degeneration pattern lesion |
| 3. Inside-Out and Outside-In models work synergistically | RCA I injection in TMEV infection | Periventricular and optic tract lesions? |
| 4. Axonal degeneration can be self-protective* | Adoptive-transfer EAE | Progressive MS? |

*Although axonal degeneration is detrimental in pathomechanisms 1 to 3, axonal degeneration can inhibit spread of neurotropic virus or toxin that is transferred via axonal flow in the central nervous system.12,26,41 Thus, axonal degeneration can be beneficial for hosts in some instances.
Thus, recruitment of inflammatory cells seems to require additional parameters, such as virus persistence in the CNS, up-regulation of adhesion molecules on lymphocytes and/or CNS endothelial cells, or CNS virus replication and axonal degeneration, both of which are seen during the acute phase of intracerebral infection of TMEV. 24

Interestingly, among mice infected with TMEV by the intracerebral route, we found some targeting of inflammatory demyelinating lesions in PBS-injected mice, although the extent of disease was milder than that in mice with RCA I injection. This suggests that changes in the CNS microenvironment may be induced by stimulation and/or minor injury to the PNS, and such stimuli can cause similar effects to the changes induced by axonal degeneration. Axonal degeneration in the CNS can activate microglia and astrocytes and can induce a variety of molecules including major histocompatibility complex (MHC) class II and cytokines such as tumor necrosis factor-α, 42,46 Peripheral nerve manipulation, such as peripheral nerve inflammation and transection, has also been shown to induce microglia and astrocyte activation with proinflammatory cytokine expression, including interleukin (IL)-1β, IL-6, and tumor necrosis factor-α in the CNS. 47–50 IL-1β, IL-6, and tumor necrosis factor-α can cause changes in BBB permeability. 51 Peripheral stimuli, such as inflammatory pain, has also been reported to increase BBB permeability. 51,52 In these models, however, neither T-cell infiltration nor demyelination in the CNS was observed during peripheral nerve inflammation. 36

In the RCA I and PBS injections, we used Fast Green FCF to help visualize the injected fluid. Fast Green FCF has been used in physiological studies because of its inertness. Although Fast Green FCF has been documented to inhibit synaptic activity, 53 various applications of the dye include staining of proteins and identification of intracerebral injection sites. 54 Neither neurotoxicity nor alterations in axonal transport of Fast Green FCF have been reported. In addition, we found that TMEV-infected mice injected with RCA I or PBS in the absence of Fast Green FCF developed similar pathology to TMEV-infected mice injected with RCA I or PBS in the presence of Fast Green FCF (data not shown). Thus, it is unlikely that Fast Green FCF itself plays a major role in recruitment of cells or inflammation.

Several factors contribute to control of lymphocyte migration into the CNS. 28 Activated T cells can enter the CNS more efficiently than naïve ones. T-cell traffic is also influenced by the activation state of the cerebral vessels. Proinflammatory cytokines can up-regulate the expression of adhesion molecules on endothelial cells. Intracranial injections of cytokines, such as interferon-γ and tumor necrosis factor-α, have been shown to recruit T cells to the injection site. 55,56 Phillips and Lampson 57 reported that local injection of interferon-γ in the CNS resulted in T-cell recruitment, which was more effective in the brainstem than in the hippocampus. They suggested that the local CNS regulatory environment, such as vasculature and antigen distribution, contributes to site-specific recruitment of T cells in the CNS.

Previously, focal inflammatory lesions have been induced by the local deposition of cytokines and thermal injury in the CNS. 58–61 Although these models allow for the induction of focal lesions in a predetermined location, the injection procedure itself, usually the needle tract, produces local changes in permeability of the BBB, the degree of MHC class II expression, and mild inflammation with inevitable axonal injury in the CNS. 58,61 In contrast, in our current model, we induced CNS microenvironment changes from the PNS, enabling us to clarify the mechanism of recruitment of inflammatory cells into the CNS without causing direct mechanical damage. We are currently investigating which factors, such as cytokines, adhesion molecules, and activation of glial cells and lymphocytes, contribute to recruitment of inflammatory cells in the CNS in our model.

In this study, we demonstrated that axonal degeneration in the CNS can target inflammatory demyelinating lesions to sites of Wallerian degeneration in a mouse model for MS. In MS, select areas within the CNS are often affected, whereas the mechanism for the preferential vulnerability of the CNS regions is unknown; for example, periventricular areas are often demyelinated in the classical form of MS, and the optic tract is involved in the opticospinal form of MS. 62 The periventricular regions can be attacked or modified after infection of the cerebrospinal fluid, whereas the optic pathway can be vulnerable to trauma or infection of the eye and optic nerve (Table 1, pathomechanism 2). Thus, axonal damage in such regions may play a role in recruiting inflammatory cells and targeting demyelinating lesions to specific sites.

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