Silencing Nogo-A Promotes Functional Recovery in Demyelinating Disease

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Objective: To determine if suppressing Nogo-A, an axonal inhibitory protein, will promote functional recovery in a murine model of multiple sclerosis (MS).

Methods: A small interfering RNA was developed to specifically suppress Nogo-A (siRNA-NogoA). The siRNA-NogoA silencing effect was evaluated in vitro and in vivo via immunohistochemistry. The siRNA was administered intravenously in 2 models of experimental autoimmune encephalomyelitis (EAE). Axonal repair was measured by upregulation of GAP43. Enzyme-linked immunosorbent assay, flow cytometry, and 3H-thymidine incorporation were used to determine immunological changes in myelin-specific T cells in mice with EAE.

Results: The siRNA-NogoA suppressed Nogo-A expression in vitro and in vivo. Systemic administration of siRNA-NogoA ameliorated EAE and promoted axonal repair, as demonstrated by enhanced GAP43+ axons in the lesions. Myelin-specific T-cell proliferation and cytokine production were unchanged in the siRNA-NogoA–treated mice.

Interpretation: Silencing Nogo-A in EAE promotes functional recovery. The therapeutic benefit appears to be mediated by axonal growth and repair, and is not attributable to changes in the encephalitogenic capacity of the myelin-specific T cells. Silencing Nogo-A may be a therapeutic option for MS patients to prevent permanent functional deficits caused by immune-mediated axonal damage.

Identifying molecules in the central nervous system (CNS) that affect functional recovery following CNS injury is essential for the development of therapeutics for multiple sclerosis (MS) and other CNS diseases. Nogo-A is a member of the reticulon family of endoplasmic reticulum anchored proteins that share a common C-terminal region. Nogo-A, also referred to as RTN4, is a potent inhibitor of neurite outgrowth and plays a critical role in negatively regulating regeneration and plasticity in the adult CNS. Nogo-A is expressed by oligodendrocytes and some neuronal subpopulations. Two regions within Nogo-A are responsible for the inhibitory effect of neurite outgrowth, one within the N-terminal Nogo-A–specific region and a second in a 66-amino acid loop in the C-terminus, referred to as Nogo-66. Nogo-66 binds the Nogo receptor (NgR), which is also the receptor for 2 myelin-associated proteins, myelin-associated glycoprotein (MAG) and oligodendrocyte-myelin glycoprotein (OMgp). Both MAG and OMgp inhibit axonal regeneration, suggesting that signaling through the NgR leads to inhibition of neurite outgrowth. In vivo administration of monoclonal antibodies specific for Nogo-A induces sprouting of Purkinje cells and enhances recovery following stroke. Similarly, in vivo administration of...
Nogo receptor antagonist peptide or monoclonal antibody enhances functional recovery in rats with spinal cord transections.\textsuperscript{3,4} Because axonal transection has been associated with permanent disability in MS,\textsuperscript{12} suppressing Nogo-A expression may promote axonal regeneration in the CNS, enhancing functional recovery. Only a few studies have evaluated the role of Nogo-A in experimental autoimmune encephalomyelitis (EAE), a model for MS. Nogo-A–deficient mice develop less severe EAE.\textsuperscript{13} This study also immunized mice with a Nogo-A peptide generating a high Nogo-A–specific antibody response prior to EAE induction, resulting in reduced incidence and severity of EAE. Interestingly, these mice had reduced inflammation, as well as a decrease in Th1-associated cytokines, suggesting that inhibiting Nogo-A may have anti-inflammatory properties. In another study, immunization with Nogo-A peptides and the subsequent generation of T cells and antibodies specific for Nogo-A appeared to be somewhat protective in EAE.\textsuperscript{14}

Our laboratory has recently been using small interfering RNAs (siRNAs) to suppress particular proteins in mice with EAE.\textsuperscript{15–18} We used a similar strategy in this study to inhibit Nogo-A. In MS and EAE, demyelination occurs due to an inflammatory response in the CNS, followed by axonal transection, leading to permanent disability.\textsuperscript{12,19–21} Because siRNAs are extremely small and have a high degree of specificity, intravenous administration causes efficient suppression of their target genes. Because there is blood–brain barrier breakdown in EAE and MS, siRNAs delivered intravenously should be able to access the site of injury. Because Nogo-A inhibits axonal elongation, silencing Nogo-A with an siRNA may provide a strategy to enhance functional recovery. This is the first study to demonstrate that inhibition of Nogo-A can improve the functional outcome in mice with established autoimmune demyelinating disease.

**Materials and Methods**

**EAE**

EAE was induced in C57Bl/6 mice by subcutaneous immunization with 200\textmu g MOG35-55 peptide emulsified in complete Freund adjuvant (CFA) with intraperitoneal injection of 200ng pertussis toxin on day 0 and 2, or in B10.PL mice by intraperitoneal injection of 10\textsuperscript{7} myelin basic protein (MBP) Ac1-11–specific T-cell receptor transgenic splenocytes.\textsuperscript{15–18}

**siRNA**

The Nogo-A and nonsense (NS) siRNA (Dharmacon, Lafayette, CO) target sequences were 5’-AAUGAUUCCGAGGC-AGAUUAU-3’ for Nogo-A and 5’-AACCAGACGAGC-CGUACACU-3’ for NS. For in vitro studies, the siRNA was transfected into N2A cells using TransIt TKO transfecting agent (Mirus, Madison, WI) as recommended by the manufacturer. For in vivo studies, 2mg/kg siRNA in phosphate-buffered saline (PBS) was injected into the tail vein.

**Lymphocyte Proliferation Assays, Enzyme-Linked Immunosorbent Assay, and Flow Cytometry**

The spleens of C57BL/6 mice were removed on day 17 or 41 after EAE induction. The splenocytes were cultured with MOG35-55 (2\mu g/ml) for 72 hours, and supernatants were collected and evaluated by enzyme-linked immunosorbent assay (ELISA) to determine interferon-\gamma (IFN\gamma) and interleukin (IL)-17 production.\textsuperscript{15–18} Infiltrating CNS lymphocytes were isolated from the CNS\textsuperscript{15} on day 17, cultured with MBP Ac1-11 overnight, and given phorbol 12-myristate 13-acetate/ionomycin for 4 hours prior to analysis. Supernatant was evaluated by ELISA, and the lymphocytes were stained for CD45, CD4, IFN\gamma, and IL-17 for flow cytometric analysis.\textsuperscript{18} For proliferation assays, splenocytes were cultured in quadruplicate with MOG35-55 or no antigen. After 72 hours, \textsuperscript{3}H-thymidine was added to the culture for an additional 18 hours, and proliferation was determined.\textsuperscript{15–18}

**Immunohistochemistry**

N2A cells were grown on cover slips, fixed with 4% paraformaldehyde, and permeabilized with 0.05% saponin. The cells were stained with anti-Nogo-A (Santa Cruz Biotechnology, Santa Cruz, CA) and Alexa Fluor 568 antirabbit immunoglobulin G (IgG), and mounted on slides with media containing 4′,6-diamidino-2-phenylindole (DAPI).

Mice were perfused with 4% paraformaldehyde, and their spinal cords were removed, post-fixed for 2 hours, and transferred to 0.2M phosphate buffer. Spinal cords were cryopreserved in 30% sucrose, frozen, and sequential longitudinal sections (10\mu m thickness) were cut, slide mounted, and stored at −20°C. For immunofluorescence, sections were rinsed with PBS and incubated in 4% bovine serum albumin/0.3%Tx-100/PBS for 1 hour at room temperature. Primary antibodies were applied for 20 hours at room temperature. Primary antibodies used were a mixture of rabbit anti-Nogo-A (1:2500; Santa Cruz Biotechnology) and rat anti-CD3 (1:100; BD Biosciences, San Jose, CA); rabbit anti-GAP43 (1:2500; Millipore, Billerica, MA) and rat anti-CD3 (1:100); or mouse antiadenomatous polyposis coli (APC; 1:500; Calbiochem, Gibbstown, NJ). Sections were rinsed with PBS, incubated with Alexa Fluor 546 goat antirabbit 1:500 and Alexa Fluor 488 antirat or Alexa Fluor 568 goat antimouse (1:500; Molecular Probes, Eugene, OR) for 1 hour at room temperature. Sections were rinsed with PBS and counterstained with DAPI.

Another set of slides were stained for neurofilament (NF; Boeringher, Mannheim, Germany) and eriochrome cyanine (EC; Sigma, St. Louis, MO). Sections were preincubated with 10% normal horse serum (NHS) followed by 1:2,000 mouse anti-NF in 5% NHS overnight at 4°C and then secondary antibody in 5% NHS. The sections were treated with 6% hydrogen peroxide in methanol followed by Elite Avidin Biotin Con-
jugate (Vector Laboratories, Burlingame, CA) and visualized with 3,3′-diaminobenzidine substrate. Slides were rinsed, treated with acetone, rinsed again, and immersed in EC solution for 30 minutes. The sections were differentiated in 5% iron alum and differentiated in borax ferricyanide. Sections were dehydrated, cleared, and coverslipped.

Quantification of immunoreactivity was performed on images acquired using confocal microscopy (510 META Scanning Laser Confocal microscope, Zeiss, Oberkochen, Germany) of sections double-labeled for Nogo-A and CD3 or Nogo-A and GAP43. Lesions were initially identified on sections using adjacent EC/NF-labeled sections. Fluorescently labeled sections were viewed by a blinded observer. CD3 immunoreactivity was used to locate white matter lesions. Images of Nogo-A and GAP43 immunoreactivity were collected within the lesion and lesion border. Images were converted to black and white, and Nogo-A and GAP43 immunoreactivity was quantified using a standardized sample box (0.001mm²) with a computerized image analysis system. A minimum of 3 lesions per animal were analyzed; lesion data for each animal were averaged, and group means for immunoreactivity were calculated.

For quantification of T cells and lesion area, 2 longitudinal sections representing 2 distinct planes of each spinal cord were stained with rat antimouse CD4 plus rat antimouse CD8 (Santa Cruz Biotechnology), FluoroMyelin Green (Invitrogen, Carlsbad, CA), and DAPI to visualize the nuclei. Each section was photographed and analyzed in a blinded fashion using an Olympus (Tokyo, Japan) EX41 microscope. Lesion was defined as the areas with a high density of nuclei, CD4+ and CD8+ cells, and loss of myelin. MicroSuite software (Olympus) was used to measure area of each lesion. CD4+ and CD8+ lymphocytes were counted throughout the entire length of each spinal cord by ImageJ (www.nih.gov).

Results

N2A cells, a neuroblastoma cell line, were transfected with a Nogo-A–specific siRNA (siRNA-NogoA) or a nonsense control siRNA (siRNA-NS, no significant sequence homology to other mouse genes). Immunocytochemistry demonstrated that Nogo-A expression was reduced by 75% in the siRNA-NogoA–transfected cells (Fig 1A, B).

It had been demonstrated that siRNA could be administered intravenously and found in most organs, with a notable exception being the CNS.22 Because the blood–brain barrier is compromised in mice with EAE, we hypothesized that siRNA could access active CNS lesions. To test this hypothesis, C57BL/6 mice were immunized with MOG35-55/CFA and administered fluorescein-labeled siRNA-NogoA intravenously after the onset of EAE. After 48 hours, the spinal cords were analyzed for the presence of fluorescein. Fluorescein was observed within the cytoplasm of cells in the lesions (see Fig 1C–G), was rarely observed outside the areas of lesions, and frequently colocalized with oligodendrocytes (as illustrated by APC staining in red; see Fig 1H). These data indicate that the blood–brain barrier breakdown in EAE allowed the siRNA access to the CNS.

To determine if suppressing Nogo-A may have a clinical benefit in immune-mediated demyelinating disease, EAE was induced by adoptive transfer in B10.PL mice (Fig 2A) or immunization of C57BL/6 mice (see Fig 2B and Table). The siRNA was administered at disease onset and 2 weeks later, which was based on a previous observation that 2 systemic doses of siRNA have a significantly longer suppression period than 1 dose.16 Within a week of the initial dose of siRNA, the siRNA-NogoA–treated mice demonstrated lower clinical scores than the control mice. After the second dose of siRNA-NogoA, mice illustrated a clinical improvement that continued for the entire monitoring period for the C57BL/6 mice. The Table illustrates the clinical data from 3 independent experiments in C57BL/6 mice. In all 3 experiments, Mann-Whitney nonparametric analysis of the individual clinical scores of each mouse in the 2 groups resulted in a statistically significant improvement with siRNA-NogoA treatment. Analysis of the individual minimum and maximum clinical scores for each mouse was reduced in the siRNA-NogoA groups (see Table). In addition, the percentage of mice that recovered from their disease, defined as improving to a score of 0 or 1 by the end of the monitoring period, was dramatically higher in the siRNA-NogoA group compared with the control group. Mice given the siRNA specific for Nogo-A at the time of disease induction, in contrast to disease onset, had a similar disease course as control mice (data not shown), supporting the hypothesis that blood–brain barrier damage is necessary for the siRNA to gain access to the CNS.

To determine if the amelioration of disease in the siRNA-NogoA mice was a result of modulating the auto-reactive T cells, the lymphocyte proliferative and cytokine responses were evaluated in the mice from the treatment experiments. There was no significant difference in antigen-specific production by splenocytes of the 2 cytokines most commonly associated with encephalitogenic T cells, IFNγ and IL-17, at day 17 or day 41 (see Fig 2). Lymphocytes isolated from the CNS on day 17 also had no difference in cytokine production by ELISA or flow cytometric analysis, indicating that siRNA-NogoA did not affect either the amount of cytokine produced or the number of cells expressing these cytokines. The anti-inflammatory cytokines, IL-4 and IL-10, were both undetectable in the splenocytes and CNS lymphocytes (data not shown). Antigen-specific proliferation was also not altered in the siRNA-NogoA–treated mice. Adoptive trans-
fer of splenocytes reactivated in vitro with antigen from C57BL/6 siRNA-NogoA–treated mice transferred disease to naive C57BL/6 recipients as effectively as splenocytes from siRNA-NS–treated mice, demonstrating that the encephalitogenic capacity of the myelin-specific cells had not been altered by suppressing Nogo-A.

Because we hypothesized that suppressing Nogo-A would have no effect on CNS inflammation or demyelination, we evaluated infiltrating T cells and lesion area. The number of T cells and lesion area were determined by staining T cells and myelin within the spinal cords of 3 mice from each treatment group at 2 time points, day 16 and day 41. Analysis of the number of infiltrating T cells and lesion area indicated that neither of these parameters was decreased in the siRNA-NogoA–treated mice (see Fig 2I–J). In fact, the number of T cells and lesion area were slightly higher in the siRNA-NogoA group at day 16, confirming that suppressing Nogo-A did not alter the inflammatory phase of the disease. As expected, the number of infiltrating T cells and lesion area were reduced by day 41 in both groups, and there was no statistically significant difference between the groups in either of these parameters, although there was a dramatic improvement in function at this time in the siRNA-NogoA–treated mice (see Fig 2B).

To confirm that the siRNA suppressed Nogo-A expression, spinal cords of 3 mice per group were analyzed by immunohistochemistry for Nogo-A expression on day 16, one week after the first dose of siRNA. Using confocal microscopy, images of the lesions (≥3 lesions per mouse) and lesion borders were acquired. As seen in Figure 3A, Nogo-A expression was low in the lesions of the siRNA-NS mice compared with the lesion borders (top 4 panels), which is unsurprising, because myelin and oligodendrocytes may be lost in the lesions. However, both the lesions and lesion borders have suppressed Nogo-A expression in the spinal cords from the mice that received siRNA-NogoA (lower 4 panels), indicating that the siRNA reduced Nogo-A expression in the areas immediately surrounding the lesions. Quantification of the Nogo-A expression demonstrated that there was a significant reduction in Nogo-A levels in the lesion borders in the siRNA-NogoA–treated mice compared with the siRNA-NS–treated mice (see Fig 3B).

We evaluated whether Nogo-A expression normal-
FIGURE 2: Silencing Nogo-A ameliorates experimental autoimmune encephalomyelitis (EAE). (A) EAE was induced by adoptive transfer of myelin basic protein (MBP)-specific T cell receptor transgenic splenocytes that were differentiated in vitro with MBP Ac1-11 (10 μg/ml) and interleukin (IL)-12 (0.5 ng/ml) into naive B10.PL mice. Small interfering RNA (siRNA)-NogoA or siRNA-nonsense (NS) (2 mg/kg) was administered intravenously on days 8 (onset of disease) and 22. (B) EAE was induced in C57BL/6 mice treated with siRNA. (C) Interferon-γ (IFN-γ) and IL-17 production measured by ELISA of CNS lymphocytes at day 17. (D) IFN-γ and IL-17 production measured by enzyme-linked immunosorbent assay (ELISA) of splenocytes at day 17. (E) IFN-γ and IL-17 production measured by ELISA of splenocytes at day 41. (G) Lymphocyte proliferation was measured by 3H-thymidine incorporation in splenocytes on day 41. (H) Splenocytes from EAE-affected C57BL/6 mice treated with siRNA-NS or siRNA-NogoA were isolated and restimulated in vitro with MOG35-55 for 72 hours and transferred into naive C57BL/6 mice. Mean clinical scores for each group (siRNA-NS, n = 8; siRNA-NogoA, n = 7) are shown. Incidence of EAE was 100%. (I, J) Longitudinal sections of spinal cords from C57BL/6 mice treated with siRNA-NS- and siRNA-NogoA-treated mice were stained for CD4 and CD8, as well as myelin. The number of T cells (G) and lesion area (H) in 2 distinct longitudinal planes was determined at 2 time points, days 16 and 41, in a blinded manner for 3 mice per group. CPM = counts per minute.

TABLE: Amelioration of EAE in C57BL/6 Mice with siRNA-NogoA

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Mice</th>
<th>Mean Day of Onset</th>
<th>Treatment Days</th>
<th>Monitoring Period</th>
<th>Mean Score</th>
<th>Recovery</th>
</tr>
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<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>siRNA-NS</td>
<td>8</td>
<td>12.75</td>
<td>14 &amp; 28</td>
<td>49 days</td>
<td>2.33 ± 0.08</td>
<td>0%</td>
</tr>
<tr>
<td>siRNA-NogoA</td>
<td>8</td>
<td>10.40</td>
<td>14 &amp; 28</td>
<td>49 days</td>
<td>1.25 ± 0.25</td>
<td>25%</td>
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<tr>
<td>Experiment 2</td>
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</tr>
<tr>
<td>siRNA-NS</td>
<td>11</td>
<td>11.80</td>
<td>9 &amp; 21</td>
<td>27 days</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>siRNA-NogoA</td>
<td>11</td>
<td>10.82</td>
<td>9 &amp; 21</td>
<td>27 days</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>Experiment 3</td>
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<tr>
<td>siRNA-NS</td>
<td>11</td>
<td>10.50</td>
<td>9 &amp; 21</td>
<td>41 days</td>
<td>2.00 ± 0.23</td>
<td>14%</td>
</tr>
<tr>
<td>siRNA-NogoA</td>
<td>11</td>
<td>12.64</td>
<td>9 &amp; 21</td>
<td>41 days</td>
<td>0.57 ± 0.30</td>
<td>71%</td>
</tr>
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</table>

*The mean ± standard error of the mean of the highest clinical score (maximum) and lowest clinical score (minimum) of each mouse after the second siRNA treatment was determined. †The percentage of mice that recovered to a clinical score of 0 or 1 by the end of the monitoring period. ‡Using Mann-Whitney analysis of the clinical scores, there was a statistically significant difference (p < 0.001) in the siRNA-NogoA–treated mice compared with the siRNA-NS mice. §Using Mann-Whitney analysis, there was a statistically significant difference in mean clinical score minimum (p = 0.007) and maximum (p = 0.018). EAE = experimental autoimmune encephalomyelitis; siRNA = small interfering RNA; NS = nonsense; ND = not determined because experiment was terminated early for ex vivo experiments.
Discussion

There are currently no therapeutic options for repairing axonal damage in MS. Although the disease is initially mediated by inflammation that damages myelin, the repeated inflammation and loss of myelin leaves axons vulnerable to damage that is irreparable, resulting in permanent functional deficits. Models of acute spinal cord injury have demonstrated that the Nogo-A pathway is amenable to therapeutic intervention that promotes axonal sprouting and elongation. However, these studies have used monoclonal antibodies and peptide inhibitors that may be neutralized by the development of antibodies. This has been a common problem with the use of recombinant interferon-β for the treatment of relapsing–remitting MS, in which neutralizing antibodies diminish the therapeutic benefit of the agent. Similarly, strategies such as induction of a strong antibody response by immunization with Nogo-A peptide, as previously explored in EAE, are not feasible in humans given the potential of generating encephalitogenic T cells. The goal of this study was to develop a therapeutic strategy that promotes functional recovery via axonal reparation with minimal adverse affects.

We have previously demonstrated that systemic administration of siRNA could be used to alter the encephalitogenic T cells and ameliorate EAE. Although it had been shown that siRNA could access most tissues when administered systemically, the CNS was not accessible. The lesions in MS are caused by blood–brain barrier breakdown initiated by inflammatory cells, and thus siRNA should be able to enter the site of CNS injury. We demonstrated that fluorescein-siRNA can enter the CNS in the lesion area and transfect cells, including oligodendrocytes. Because fluorescein is quite large relative to the siRNA, the number of fluorescein particles probably under-represents the amount of siRNA molecules that enter the CNS cells.

More importantly, we found that administration of a Nogo-A siRNA improved the functional capacity of mice with EAE, which was associated with decreased Nogo-A expression. In addition, GAP43, a protein expressed by growing and sprouting axons, was significantly upregulated, suggesting that axonal repair may be responsible for the improved clinical outcome in the siRNA-
NogoA–treated mice. Because a previous study indicated that blocking Nogo-A with antibodies in EAE resulted in less CNS inflammation and found less Th1-associated cytokines, we investigated whether silencing Nogo-A altered the phenotype of myelin-specific T cells. However, we found no change in T-cell proliferation or cytokine production in the siRNA-NogoA–treated mice. Transfer of myelin-specific T-cells from the siRNA-NogoA and control mice into naive recipient mice resulted in similar EAE courses, indicating that the encephalitogenic T cells had not been altered by the siRNA-NogoA treatment. A recent study analyzing NgR expression in human lymphocytes found that although B cells, T cells, and monocytes express NgR1 in a regulated fashion, an inhibitory Nogo-A peptide failed to alter proliferation or cytokine production by human lymphocytes. This study also found that myelin could regulate human T-cell motility, but this study targeted NgR and not Nogo-A, one of several ligands of the NgR. Analysis of T cells and lesion area indicated that suppression of Nogo-A did not reduce T-cell migration into the CNS or reduce lesion size. Thus, the data indicate that suppression of Nogo-A promoted functional recovery by promoting axonal repair.

This is the first study to demonstrate that siRNA directed toward axonal inhibitors expressed in the CNS is a promising therapeutic option for promoting functional recovery. Historically, 50% of MS patients with relapsing–remitting MS progressed to secondary progressive MS within 10 years of diagnosis. The use of immunomodulatory therapies has slowed disease progression, but ultimately the majority of MS patients develop permanent functional deficits due to axonal damage. Thus, the development of therapies that may protect axons or promote axonal growth is critical for treating secondary progressive MS, as well as the 10% of MS patients with primary progressive MS.

Acknowledgment
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Potential Conflicts of Interest
Nothing to report.
A

Mouse A

CD3

GAP43

Day 16

siRNA-NS

siRNA-NogoA

Day 41

siRNA-NS

siRNA-NogoA

B

Target Area [mm²]

p=0.0031

p=0.0025

NS

NogoA

Day 16

Day 41

References


FIGURE 5: Silencing Nogo-A induces upregulation of GAP43. Three C57BL/6 mice per group were perfused on days 16 (7 days after 1st small interfering RNA [siRNA] treatment) and 41 postimmunization (20 days after 2nd siRNA treatment). (A) The spinal cords were sectioned longitudinally and stained for GAP43 (red) and CD3 (green). Confocal microscopy was used to image lesions for each spinal cord. Two images of lesions from 2 different mice (mouse A and B) are shown for each treatment group. White dotted lines represent lesion border. (B) The amount of GAP43 immunoreactivity was quantified using a standardized sample box (0.001mm²) with a computerized image analysis system. A minimum of 3 lesions per animal were analyzed; lesion data for each animal were averaged, and group means for immunoreactivity were calculated and compared. NS = nonsense.


