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Citation for published version:

Miron, VE, Zehntner, SP, Kuhlmann, T, Ludwin, SK, Owens, T, Kennedy, TE, Bedell, BJ & Antel, JP 2009, 'Statin therapy inhibits remyelination in the central nervous system' *American Journal Of Pathology*, vol 174, no. 5, pp. 1880-90. DOI: 10.2353/ajpath.2009.080947

Digital Object Identifier (DOI):

[10.2353/ajpath.2009.080947](https://doi.org/10.2353/ajpath.2009.080947)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Publisher's PDF, also known as Version of record

Published In:

American Journal Of Pathology

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Statin Therapy Inhibits Remyelination in the Central Nervous System

Veronique E. Miron,^{*†} Simone P. Zehntner,[‡]
Tanja Kuhlmann,[§] Samuel K. Ludwin,[¶]
Trevor Owens,^{||} Timothy E. Kennedy,[†]
Barry J. Bedell,[‡] and Jack P. Antel^{*}

From the Neuroimmunology Unit,^{*} the Centre for Neuronal Survival,[†] and the Brain Imaging Center,[‡] Montreal Neurological Institute, McGill University, Montreal, Quebec, Canada; the Institute of Neuropathology,[§] University Hospital, Munster, Germany; the Department of Neuropathology,[¶] Queen's University, Kingston, Ontario, Canada; and the Centre for Medical Biotechnology,^{||} Institute of Medical Biology, University of Southern Denmark, Denmark

Remyelination of lesions in the central nervous system contributes to neural repair following clinical relapses in multiple sclerosis. Remyelination is initiated by recruitment and differentiation of oligodendrocyte progenitor cells (OPCs) into myelinating oligodendrocytes. Simvastatin, a blood-brain barrier-permeable statin in multiple sclerosis clinical trials, has been shown to impact the *in vitro* processes that have been implicated in remyelination. Animals were fed a cuprizone-supplemented diet for 6 weeks to induce localized demyelination in the corpus callosum; subsequent return to normal diet for 3 weeks stimulated remyelination. Simvastatin was injected intraperitoneally during the period of coincident demyelination and OPC maturation (weeks 4 to 6), throughout the entire period of OPC responses (weeks 4 to 9), or during the remyelination-only phase (weeks 7 to 9). Simvastatin treatment (weeks 4 to 6) caused a decrease in myelin load and both Olig2^{strong} and Nkx2.2^{strong} OPC numbers. Simvastatin treatment (weeks 4 to 9 and 7 to 9) caused a decrease in myelin load, which was correlated with a reduction in Nkx2.2^{strong} OPCs and an increase in Olig2^{strong} cells, suggesting that OPCs were maintained in an immature state (Olig2^{strong}/Nkx2.2^{weak}). NogoA+ oligodendrocyte numbers were decreased during all simvastatin treatment regimens. Our findings suggest that simvastatin inhibits central nervous system remyelination by blocking progenitor differentiation, indicating the need to monitor

effects of systemic immunotherapies that can access the central nervous system on brain tissue repair processes. (*Am J Pathol* 2009, 174:1880–1890; DOI: 10.2353/ajpath.2009.080947)

Multiple sclerosis (MS) is characterized by inflammatory demyelinating lesions in the central nervous system (CNS). Remyelination of such lesions, recognized by histopathological and *in vivo* imaging techniques, is considered to contribute to neural repair following clinical relapses.^{1,2} Experimental models of CNS demyelination indicate that remyelination is mediated by oligodendrocyte progenitor cells (OPCs), and requires their migration into the lesion and differentiation into mature myelinating phenotypes.³ OPCs have been identified in the human adult brain and surrounding demyelinated MS lesions.⁴ The impact of any therapeutic agent on OPCs and remyelination may be a significant determinant of long-term neurological function.

Simvastatin, a blood-brain barrier-permeable statin,⁵ inhibits 3-hydroxy-3-methylglutaryl co-enzyme A reductase, restricting synthesis of cholesterol and the post-translational lipid attachments, isoprenoids. The beneficial action of simvastatin in reducing initial disease severity in an animal model of MS, experimental autoimmune encephalomyelitis,⁶ has propelled this agent into MS clinical trials.⁷ The reported increase in myelin repair in experimental autoimmune encephalomyelitis following short-term statin therapy⁸ could reflect an indirect consequence of immunomodulatory effects or a direct impact on oligodendroglia. We have previously reported that short-term statin treatment of human and rodent OPCs and mature oligodendrocytes (OLGs) *in vitro* induces process outgrowth and differentiation via isoprenoid depletion.⁹ Other studies have demonstrated that

Supported by a grant from the Foundation of the Multiple Sclerosis Society of Canada (J.P.A. and T.E.K.), the National Multiple Sclerosis Society (NMSS; JPA), and the Canadian Institutes of Health Research (studentship to V.E.M.).

Accepted for publication January 13, 2009.

Address reprint requests to Dr. Jack P. Antel, Room 111, Neuroimmunology Unit, Montreal Neurological Institute, 3801 University, Montreal, QC, Canada, H3A 2B4. E-mail: jack.antel@mcgill.ca.

statin treatment of adult human brain-derived OPCs also enhances their differentiation while inhibiting proliferation.¹⁰ Prolonged exposure to statins, however, can cause oligodendroglial process retraction through cholesterol depletion, and cell death by blocking synthesis of isoprenoids and cholesterol.⁹

Given the multiple *in vitro* effects of statins on progenitor cell responses that are presumed to be implicated in remyelination, the objective of this study was to assess whether these observations translated into effects on remyelination *in vivo*. In our current study we used oral cuprizone administration as an *in vivo* model of non-immune system-initiated, brain-localized demyelination and remyelination.^{11,12} This model allowed us to determine the direct effect of long-term statin therapy on remyelination, independent of its indirect effects mediated via systemic immune modulation. The massive demyelination in specific white matter tracts that ensues from oral cuprizone administration is reproducible and localized, facilitating assessment of effects of pharmacological treatments on acceleration or inhibition of remyelination.¹³ The concomitant demyelination and OPC responses/remyelination observed with cuprizone administration replicates what has been observed in demyelinating MS lesions.¹⁴ This model also provides the opportunity to study remyelination alone once the cuprizone toxin is removed from the diet.¹³ Given the role of OPCs in the remyelination process, we evaluated effects of simvastatin on progenitors using the transcription factors Olig2 and Nkx2.2 as markers, and identified mature OLGs by NogoA expression. These same markers have been used to identify OPCs and OLGs in both MS lesions and normal adult brain.¹⁵ The cuprizone model avoids confounding issues such as axonal damage, blood-brain barrier breakdown, and moderate traumatic injury with consequent immune cell infiltration at the injection site.¹⁶⁻¹⁸

We elected to use simvastatin due to its use in MS clinical trials,⁷ its ability to cross the blood-brain barrier,⁵ and the finding that chronic oral administration of simvastatin in mice results in significant concentrations of statin in the brain with resultant changes in gene expression.¹⁹ We report that simvastatin significantly inhibits the robust remyelination that normally occurs subsequent to CNS demyelination from oral administration of the oligodendrocyte toxin, cuprizone.

Materials and Methods

Animals

Animal handling and experiments were conducted according to the Canadian Council on Animal Care guidelines and were approved internally by the McGill University Animal Care Committee. Fourteen-week-old adult male C57BL/6J mice (Jackson Laboratories; Bar Harbor, ME) were fed 0.2% cuprizone-supplemented chow *ad libitum* (bis-cyclohexane oxaldihydrazone; Sigma, Oakville, CA; Harlan Teklad, Madison, WI) for weeks 1 to 6, to induce a reproducible and localized OLG cell death and demyelination in the corpus callosum. Remyelination in

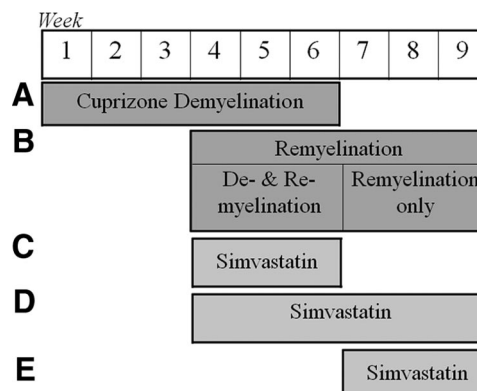


Figure 1. Treatment protocol. Cuprizone was administered for weeks 1 to 6 to induce demyelination in the corpus callosum (CC). **A:** OPC proliferation/maturation in the CC begins at week 4 of cuprizone treatment, during concomitant demyelination. Once animals are returned to normal diet (week 7), robust remyelination occurs (weeks 7 to 9, **B**). Simvastatin (2 mg/kg/day) was injected daily during the period of concomitant demyelination and OPC maturation (weeks 4 to 6, **C**), during the entire remyelination period (weeks 4 to 9, **D**) or during the remyelination only phase (weeks 7 to 9, **E**). As controls, animals on normal diet received simvastatin therapy from weeks 4 to 6 or 4 to 9.

cuprizone-treated mice was achieved by returning the mice to a normal diet for three subsequent weeks (weeks 7 to 9). OPC proliferation, recruitment, and differentiation are observed before return to normal diet (week 4 of treatment).¹³ A subset of mice received daily i.p. injections of 2 mg of simvastatin/kg of body weight (Calbiochem, San Diego, CA) or vehicle (dimethyl sulfoxide) dissolved in sterile 0.9% sodium chloride solution. We administered the active metabolite of the drug thereby bypassing the need for oral ingestion/metabolism; both orally administered simvastatin that has been metabolized, and the injected active metabolite, have the potential to be partially reverted back to the parent drug in tissue to the same extent.²⁰ Simvastatin was administered for weeks 4 to 6 during the period of concomitant demyelination and remyelination, from weeks 4 to 9 during the entire period of OPC responses, or during the remyelination-only period (weeks 7 to 9) (Figure 1). Of cuprizone-treated animals, nine were treated with simvastatin and nine with vehicle. Of the animals on normal diet, six were treated with simvastatin and six with vehicle. When assessing myelin levels in the corpus callosum, there was no significant difference between animals treated with cuprizone alone and those treated with cuprizone and the vehicle used to reconstitute the simvastatin (data not shown), demonstrating that the vehicle did not have any impact on myelin processes in cuprizone-fed animals. No adverse side effects or gross behavioral abnormalities were observed in any treatment group throughout the experiments.

Immunohistochemistry

Animals were anesthetized with ketamine (125 mg/kg)-xylazine (25 mg/kg) and exsanguinated by intracardial perfusion with PBS. Brains were extracted and fixed in 10% neutral-buffered formalin, dehydrated through graded alcohols and xylene, and subsequently embedded in

paraffin wax. Coronal brain sections of 5 μm were de-waxed and rehydrated. Four sections from each animal were stained with luxol fast blue (LFB) overnight in a warm humid incubator, rinsed with 95% ethanol, lithium carbonate, 70% ethanol, and water. Sections were counterstained with H&E and subsequently dehydrated. Immunohistochemistry staining was performed using a Lab Vision Autostainer 360 (LabVision, Fremont, CA) after rehydration and heat-induced antigen retrieval in boiling 10 mmol/L citrate buffer pH 6.1 (LabVision) or EnVision retrieval buffer pH 6 (Dako, Mississauga, Ontario, Canada) at elevated pressure. All samples were treated for endogenous peroxidase activity with 0.3% H_2O_2 followed by blocking with LVBBlock (LabVision). Sections were incubated with the appropriate antibodies: mouse anti-myelin basic protein (MBP; Sternberger monoclonals, Lutherville, MD), rabbit anti-gial fibrillary acidic protein (GFAP; Medicorp, Montreal, Quebec, Canada), rabbit anti-ionized calcium-binding adaptor molecule-1 (IBA-1; WAKO Chemicals USA, Richmond, VA), rabbit anti-Olig2 (IBL, Gunma, Japan), mouse anti-Nkx2.2 (clone 74.5A5; Developmental Studies Hybridoma Bank, University of Iowa, IA), and rabbit anti-neurite outgrowth inhibitor protein A (NogoA; Chemicon, Temecula, CA). Primary antibodies were detected using avidin-biotin (LabVision), LV polymer (LabVision), or EnVision (Dako) amplification, followed by visualization with horseradish peroxidase-catalyzed 3-amino-9-ethylcarbazole (LabVision) chromogen deposition. Control sections showed low non-specific staining. All sections were blindly digitized using a Nikon Eclipse 55i microscope (Nikon Canada, Mississauga, ON, Canada) equipped with a QICAM 12-bit Fasz 1394 digital camera (QImaging, Surrey, BC, Canada). Automated high-resolution whole-section imaging was performed using Mirax Scan (Carl Zeiss Microimaging, Inc., Gottingen, Germany).

Controls for Simvastatin Biological Activity

We verified the ability of simvastatin to decrease cholesterol levels *in vivo* by measuring serum cholesterol levels. Blood was collected from the mice before perfusion and allowed to coagulate on ice for a few hours. Serum was collected and homogenized with chloroform-methanol (2:1), solutions were centrifuged for 10 minutes at 10,000 rpm, and the organic phase was isolated and centrifuged. Solvent was evaporated overnight, and pellets of concentrated cholesterol were re-dissolved in reaction buffer. Cholesterol levels were assessed using the Amplex Cholesterol Assay (Molecular Probes, Eugene, OR) according to the manufacturer's instructions.

Analysis

LFB staining of the medial corpus callosum was blindly assessed for demyelination through the microscope. Scores were assigned as follows: 0 to fully myelinated sections, 1 to sections with mild demyelination, 2 to sections with scarce myelin, 3 for completely demyelinated medial corpus callosum, and 4 for demyelination of me-

dial and more lateral corpus callosum. A similar scoring system has been previously used to assess LFB histochemical stains.¹² The area of MBP immunohistochemical staining was assessed objectively using an optical density image (Scion Image software) calibrated to $\times 20$ objective image. OPCs (Olig2^{strong}, Nkx2.2^{strong}), OLGs (NogoA+), astrocytes (GFAP+), and microglia (IBA-1+) were blindly counted in the medial corpus callosum in $\times 10$ to $\times 20$ objective images using a calibrated grid with Adobe Photoshop software (San Jose, CA). Data are represented as average score/number of immunopositive cells per mm^2/area of MBP staining (μm^2) \pm SEM. All comparisons between conditions were performed using one-way analysis of variance followed by Newman-Keuls multiple comparison posthoc test. Normality was assessed using Bartlett's test for equal variances. Probability values ≤ 0.05 were considered statistically significant.

Results

Simvastatin Is Functionally Active

The dose of statin used in our study was in the same range as used in previous experimental immunomodulatory directed studies.^{6,8} Statin was confirmed to be functionally active *in vivo* by the measure of serum cholesterol levels. We observed a significant decrease following long-term simvastatin treatment of animals on normal diet (weeks 4 to 9, 7.9 $\mu\text{g}/\text{ml}$) relative to vehicle-treated control (139.8 $\mu\text{g}/\text{ml}$). This was also noted when simvastatin was administered to cuprizone-fed animals (weeks 4 to 9, 17.8 $\mu\text{g}/\text{ml}$; weeks 7 to 9, 34.1 $\mu\text{g}/\text{ml}$) as compared with cuprizone alone (87.6 $\mu\text{g}/\text{ml}$).

Effect of Simvastatin Treatment on Myelin Load

To assess the effect of simvastatin on remyelination and myelin maintenance in the cuprizone model (Figure 1), demyelination in the medial corpus callosum (CC) was scored following LFB histochemistry, and myelin load in the medial CC was quantitatively assessed by determining the area of staining of MBP in digitized immunohistochemical sections.

Long-Term Simvastatin Treatment Hampers Myelin Maintenance under Non-Demyelinating Conditions

We first determined whether simvastatin could impact ongoing myelin maintenance processes under non-demyelinating conditions. Short-term simvastatin therapy alone (weeks 4 to 6) did not induce any notable effects on demyelination/myelin load in animals on normal diet (Figure 2B). However, long-term simvastatin therapy alone given to animals on normal diet (weeks 4 to 9) did significantly decrease LFB staining (Figure 2A), increase demyelination score (Figure 2C), and reduce MBP levels (Figure 2, A and D) relative to vehicle-treated control, indicative of diminished myelin content.

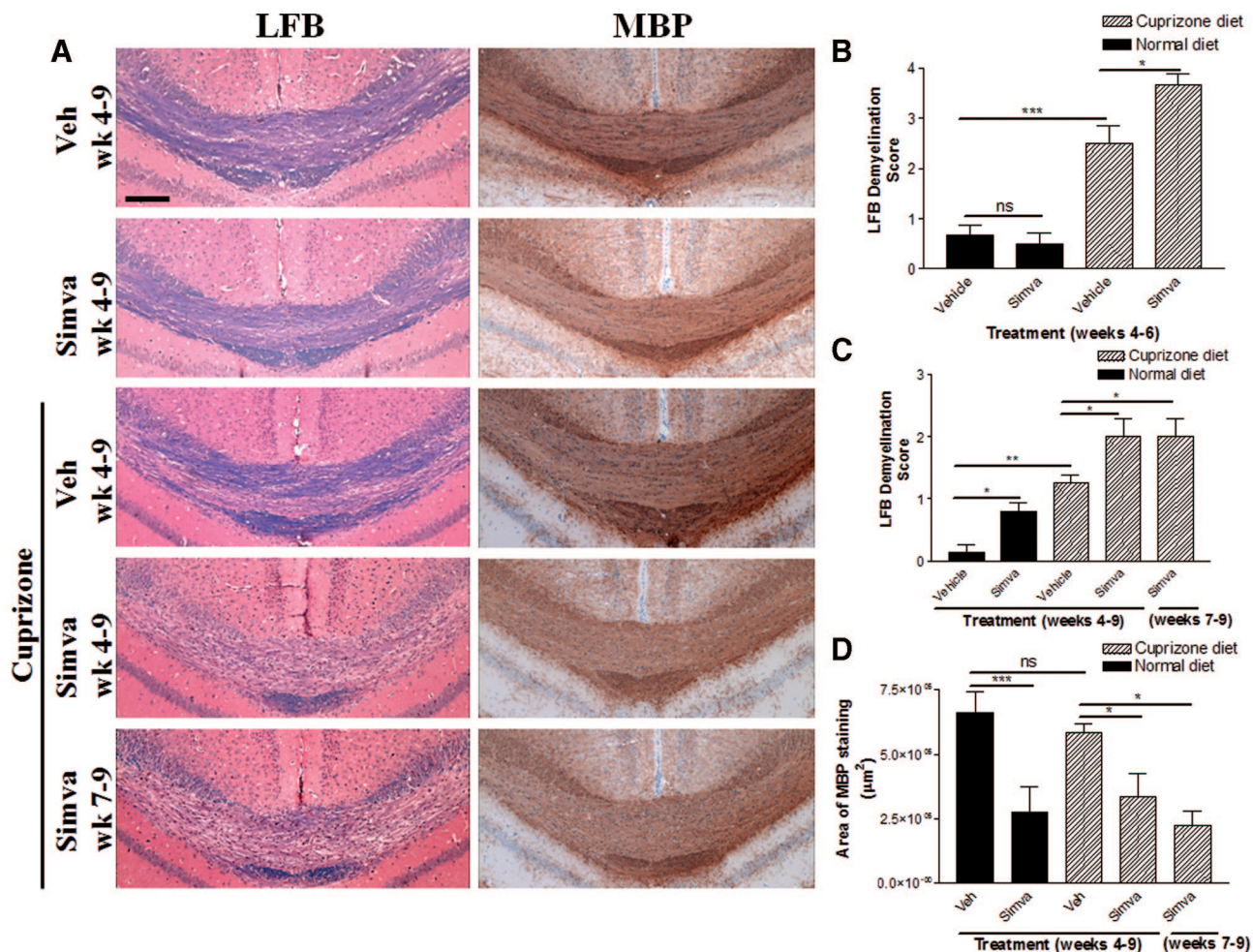


Figure 2. Simvastatin inhibits remyelination in cuprizone-demyelinated corpus callosum. **A:** Luxol fast blue (LFB) histochemistry (left panels) and myelin basic protein (MBP) immunohistochemistry (right panels) were used to evaluate myelin content in the corpus callosum (CC). Representative images of sections of animals treated with vehicle (Veh) or simvastatin (Simva) for weeks 4 to 9 or weeks 7 to 9. Scale bar = 200 μm. **B:** Average LFB demyelination scores (± SEM) for animals treated with vehicle or simvastatin during weeks 4 to 6. By week 6, simvastatin treatment of animals on normal diet (black bars) had no effect on myelin load relative to vehicle control. Cuprizone administration (gray bars) significantly increased demyelination score relative to vehicle-treated animals on normal diet (black bars). Simvastatin treatment of cuprizone-fed animals further increased demyelination scores. **C:** Average LFB demyelination scores (± SEM) for animals treated with vehicle or simvastatin for weeks 4 to 9 or 7 to 9. Long-term simvastatin treatment of animals on normal diet (black bars) significantly increased demyelination score. Cuprizone treated animals (gray bars) injected with vehicle still demonstrated an overall increase in demyelination at week 9 relative to control, yet some recovery was observed as compared with week 6 (**B**). Simvastatin treatment (weeks 4 to 9, 7 to 9) produced persistent increases in demyelination scores relative to cuprizone alone. **D:** Average area of MBP staining in the CC (μm² ± SEM) for animals treated with vehicle or simvastatin during weeks 4 to 9 or 7 to 9. Simvastatin treatment of animals on normal diet (black bar) caused a significant reduction in area of MBP staining relative to vehicle control. By week 9, MBP levels in the CC of animals treated with cuprizone (gray bar) and vehicle had recovered to those observed in animals on normal diet. Simvastatin treatment (weeks 4 to 9, 7 to 9) of cuprizone-fed animals resulted in a reduced area of MBP staining relative to cuprizone alone. Analysis of variance *P* values <0.001 for LFB scores and 0.0019 for MBP quantification. ns *P* > 0.05, **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

Simvastatin Treatment Decreases Myelin Load during Concomitant Demyelination and Initial OPC Proliferation/Maturation

As expected, 6 weeks of oral cuprizone treatment (Figure 1A) decreased LFB staining and increased the demyelination score in the medial CC in comparison with animals on normal diet (Figure 2B). Simvastatin treatment during weeks 4 to 6, ie, the period of concomitant demyelination and OPC proliferation/maturation¹³ (Figure 1, B and C), induced an additional decrease in LFB staining and increase in demyelination score, relative to cuprizone-treated animals that were administered only the vehicle used to dilute simvastatin (Figure 2B), suggesting either enhanced demyelination or inhibition of initial OPC responses by simvastatin. This was addressed by deter-

mining the impact of simvastatin on the post-cuprizone remyelination phase, discussed below.

Simvastatin Treatment Impedes Post-Cuprizone Remyelination

On return to normal diet for 3 weeks subsequent to cuprizone administration (Figure 1B, week 9), there was an increase in LFB staining (Figure 2A), a significant decrease in demyelination score (*P* < 0.05, Figure 2B), and an increase in area of MBP staining in the medial CC (Figure 2, A and D) when compared with immediately post-cuprizone administration (week 6), indicative of remyelination.¹³ Treatment with simvastatin either during the entire period of OPC responses (weeks 4 to 9, Figure

1D) or during the remyelination-only phase (weeks 7 to 9, Figure 1E) was associated with a decrease in LFB staining (Figure 2A), significant increase in demyelination score (Figure 2C), and decrease in area of MBP staining (Figure 2, A and D) relative to vehicle-treated animals, thereby indicating reduced myelin content and inhibition of remyelination by simvastatin.

Effects of Simvastatin Treatment on Oligodendrocyte Progenitor Cells

Given the role of OPCs in the remyelination process, we evaluated the effects of simvastatin on oligodendroglial cells, by identifying OPCs by high expression levels ('strong') of the oligodendrocyte specification transcription factors Olig2 and Nkx2.2,^{15,21,22} and determined the average number of strongly positive cells in the medial CC. In the normal CC, both Olig2^{strong} and Nkx2.2^{strong} OPCs followed a random distribution pattern (Figure 3, A and B).

Simvastatin Treatment Influences Progenitor Numbers and Differentiation State under Non-Demyelinating Conditions

Animals on a normal diet demonstrated a significant increase in Olig2^{strong} cells ($P < 0.01$, Figure 3C, Figure 4, A and B) and a significant decrease in Nkx2.2^{strong} cells ($P < 0.001$, Figure 3D, Figure 4, A and B) at week 9 as compared with week 6. Since Olig2^{strong}/Nkx2.2^{weak} cells are regarded as immature OPCs²²; these findings may reflect continuous infiltration of immature OPCs into the normal adult white matter. These cells may eventually contribute to the mature OLG pool over time, as continuous replacement of OLGs has been observed in normal adult CNS.^{23,24} Both short-term and long-term simvastatin treatment of animals on normal diet (weeks 4 to 6, 4 to 9) caused an increase in the numbers of Olig2^{strong} (Figure 3C, Figure 4, A and B) and Nkx2.2^{strong} cells (Figure 3D, Figure 4, A and B) relative to vehicle-treated controls. This may suggest that simvastatin treatment caused OPCs to be maintained in a pre-OLG state (Olig2^{strong} and Nkx2.2^{strong}).²² These cells did not contribute to the OLG pool or myelin maintenance as a decrease in myelin content was observed with long-term simvastatin treatment.

Simvastatin Treatment Reduces Progenitor Numbers during Concomitant Demyelination and Progenitor Infiltration

Cuprizone administration for 6 weeks caused an increase in Olig2^{strong} cells (Figure 3C, Figure 4A) relative to animals on normal diet; an increase in these cells has been previously observed in demyelinated lesions in the adult CNS.²¹ This increase was not associated with a simultaneous increase in Nkx2.2^{strong} cells (Figure 3D, Figure 4A). There was a strong correlation between demyelination score and average numbers of Olig2^{strong}

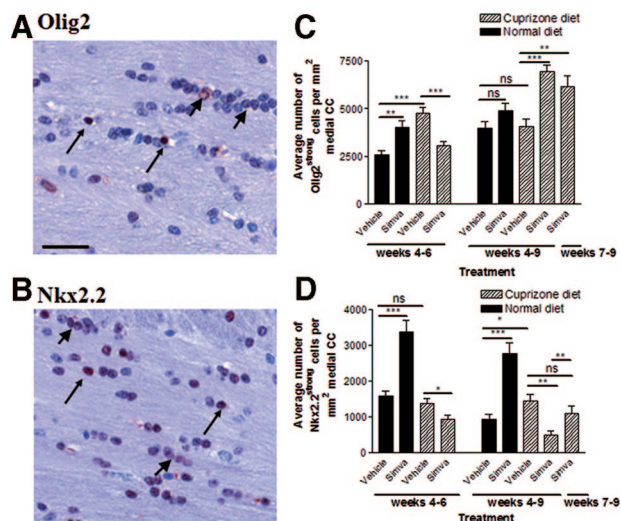


Figure 3. Quantification of the impact of simvastatin on OPCs in the corpus callosum. **A, B:** Coronal sections of corpus callosum of normal animals on normal diet. OPCs (small arrowheads) were identified as having strong staining for the transcription factors Olig2 and Nkx2.2 and showed a random distribution pattern (brown). Weakly positive cells (large arrowheads) were taken to be mature OLGs and were aligned in rows. Nuclei were labeled with hematoxylin (blue). Scale bar = 25 μ m. **C:** Average numbers of Olig2^{strong} OPCs per mm² medial corpus callosum (CC) \pm SEM. Simvastatin treatment (Simva) (weeks 4 to 6) of animals on normal diet (black bars) caused a significant increase in Olig2^{strong} OPCs relative to vehicle control. Cuprizone administration (gray bars) resulted in an increase in Olig2^{strong} cells at this time. Simvastatin treatment during weeks 4 to 6 inhibited this increase in Olig2^{strong} OPCs in the CC. At week 9, vehicle treated animals on normal diet (black bars) show an increase in Olig2^{strong} cells compared with week 6. At this time point, simvastatin treatment of animals on normal diet had no significant effect on numbers of Olig2^{strong} OPCs. Cuprizone-treated animals showed a decrease in these cells by week 9 relative to week 6. However, simvastatin treatment during weeks 4 to 9 or 7 to 9 caused a significant increase in Olig2^{strong} OPCs in the CC relative to cuprizone alone. **D:** Average numbers of Nkx2.2^{strong} OPCs per mm² medial CC \pm SEM. Simvastatin treatment (weeks 4 to 6) of animals on normal diet (black bars) caused a significant increase in Nkx2.2^{strong} cells in comparison with control (vehicle + normal diet). Simvastatin treatment (weeks 4 to 6) of animals on cuprizone (gray bars) was associated with a decrease in Nkx2.2^{strong} OPCs in the CC versus control (cuprizone + vehicle). By week 9, vehicle treated animals on normal diet (black bars) showed a decrease in Nkx2.2^{strong} cells compared with week 6. At this time point, simvastatin treatment of animals on normal diet caused an increase in numbers of Nkx2.2^{strong} OPCs. Cuprizone administration induced a significant increase in Nkx2.2^{strong} OPCs by week 9. Simvastatin treatment (weeks 4 to 9) of animals on cuprizone caused a significant decrease in numbers of Nkx2.2^{strong} OPCs in the CC relative to cuprizone alone. Analysis of variance P values < 0.001 . ns $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

cells in the CC ($R^2 = 0.82$), and a weaker correlation with numbers of Nkx2.2^{strong} cells ($R^2 = 0.66$) at the end of the demyelination period (week 6). Our results suggest that immature OPCs (Olig2^{strong}/Nkx2.2^{weak}) infiltrated the CC in response to demyelination, a step required before the initiation of remyelination. Short-term statin treatment (weeks 4 to 6) of animals on cuprizone caused a significant decrease in Olig2^{strong} OPCs (Figure 3C, Figure 4A) and Nkx2.2^{strong} cells (Figure 3D, Figure 4A), implying that reduced progenitor numbers may have contributed to the diminishment in initial remyelination observed with this treatment regimen.

Simvastatin Treatment Influences Progenitors during the Post-Cuprizone Remyelination Phase

Animals that were administered a normal diet during weeks 7 to 9 (remyelination phase) subsequent to cupri-

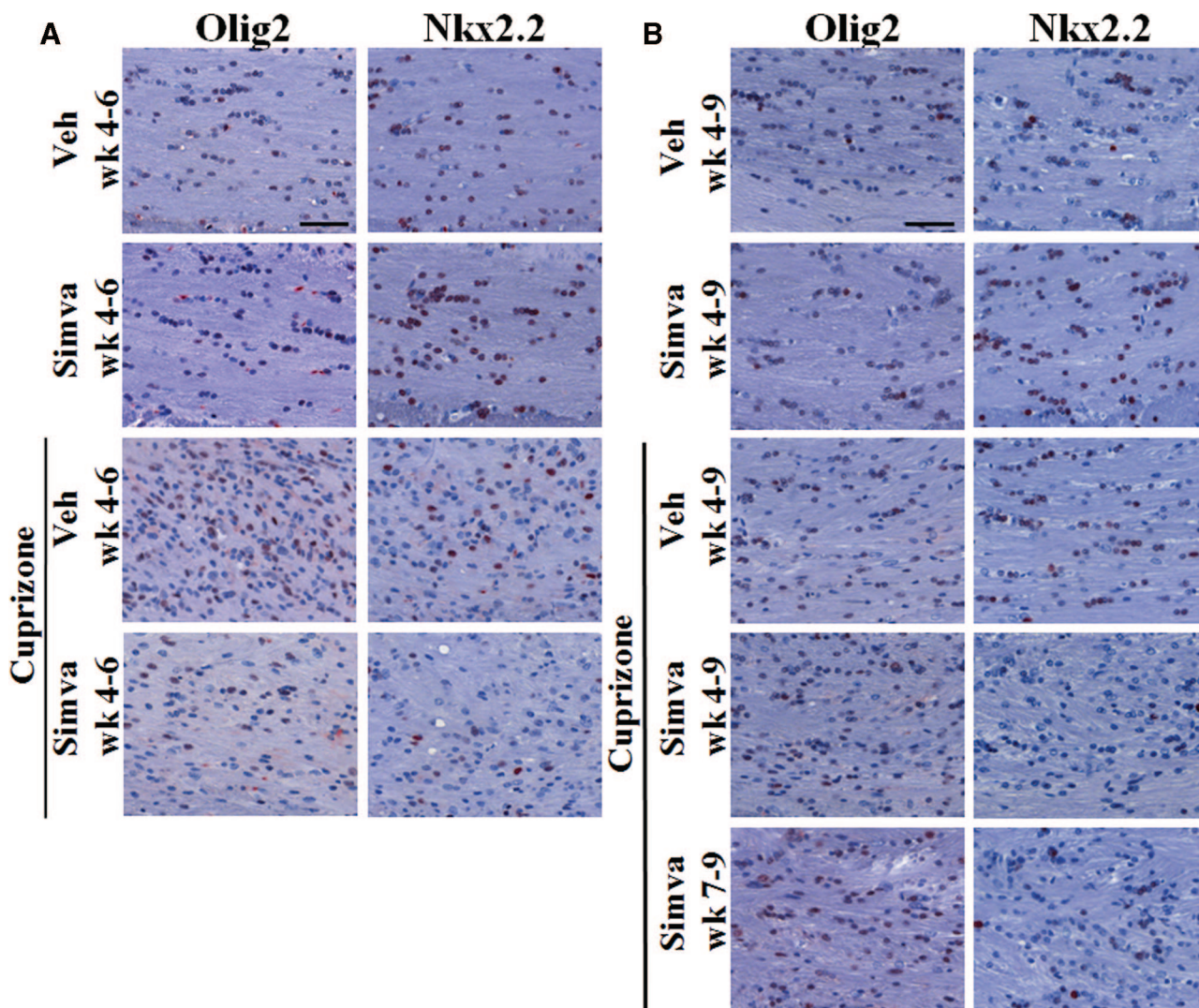


Figure 4. Simvastatin impacts OPCs in the cuprizone-demyelinated corpus callosum. **A:** Coronal sections of corpus callosum of animals sacrificed at week 6. OPCs were identified by strong immunostaining against Olig2 (left panels) and Nkx2.2 (right panels) (brown). Nuclei were labeled with hematoxylin (blue). Simvastatin treatment (weeks 4 to 6) of animals on normal diet caused an increase in Olig2^{strong} OPCs and Nkx2.2^{strong} OPCs relative to vehicle control. Cuprizone-treated animals demonstrated an increase in Olig2^{strong} cells but not Nkx2.2^{strong} OPCs at this time. These animals also demonstrated an increase in total nuclei in the corpus callosum relative to animals on normal diet, which represented astroglial and microglial infiltration (addressed in Figure 7). Simvastatin treatment of animals on a cuprizone diet during weeks 4 to 6 caused a relative decrease in Olig2^{strong} and Nkx2.2^{strong} OPCs relative to cuprizone-vehicle controls. Scale bar = 50 μ m. **B:** Coronal sections of corpus callosum of animals sacrificed at week 9. Simvastatin treatment of animals on normal diet had no effect on numbers of Olig2^{strong} OPCs but caused an increase in numbers of Nkx2.2^{strong} OPCs relative to vehicle controls. Cuprizone-treated animals showed a decrease in Olig2^{strong} OPCs and an increase in Nkx2.2^{strong} OPCs at week 9 relative to week 6. However, simvastatin treatment during weeks 4 to 9 or 7 to 9 caused a significant increase in Olig2^{strong} OPCs and decrease in Nkx2.2^{strong} OPCs relative to cuprizone alone. Scale bar = 50 μ m.

zone treatment showed a trend toward a decrease in Olig2^{strong} cells relative to the demyelination period (Figure 3C, Figure 4B), a phenomenon previously documented by others in remyelinating lesions.²¹ A trend toward an increase in Nkx2.2^{strong} cell numbers at the end of the remyelination period was also observed in these animals (week 9; Figure 3D, Figure 4B). OPCs increase Nkx2.2 expression and decrease Olig2 levels in association with terminal differentiation^{15,22} a requirement for remyelination to occur. Simvastatin administration during the entire period of OPC responses (weeks 4 to 9) or during the remyelination-only phase (weeks 7 to 9) significantly increased Olig2^{strong} cells. The more prolonged simvastatin treatment regimen (weeks 4 to 9) also induced a significant decrease in Nkx2.2^{strong} cells relative

to cuprizone-vehicle controls, whereas the shorter treatment regimen from weeks 7 to 9 did not (Figure 3D, Figure 4B). This observation suggests that either simvastatin slowed the migration of Olig2^{strong} OPCs into the CC such that they were only increased by week 9, that continued myelin injury recruited more Olig2^{strong} OPCs to the CC, or that OPCs were maintained in an immature state (Olig2^{strong}/Nkx2.2^{weak}) from simvastatin exposure.

Effects of Simvastatin Treatment on Mature Oligodendrocytes

Mature OLG cell bodies were identified by expression of NogoA, a marker previously demonstrated to reliably la-

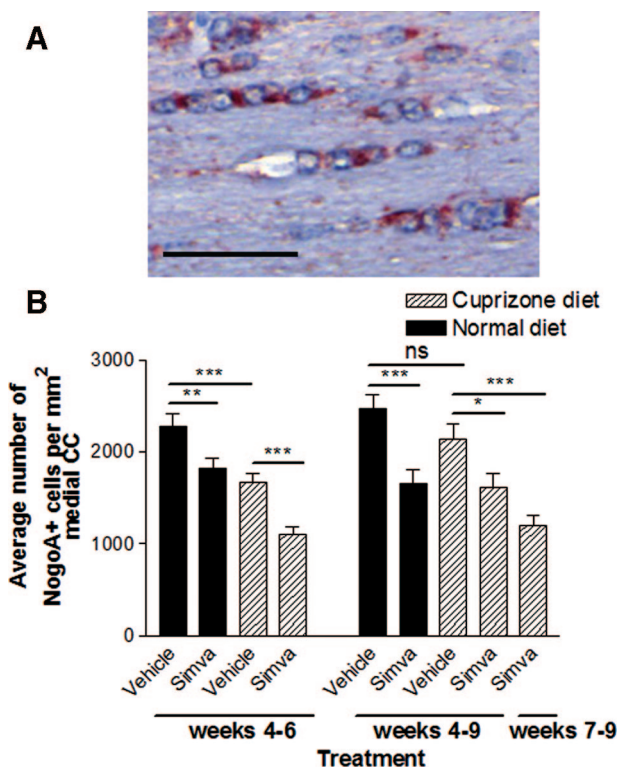


Figure 5. Quantification of the effect of simvastatin on mature OLGs in the corpus callosum. **A:** Mature OLG cell bodies were labeled with a NogoA-directed antibody (brown) and nuclei were labeled with hematoxylin (blue). Scale bar = 50 μ m. **B:** Average numbers of NogoA+ cells per mm² of the medial corpus callosum (CC) (\pm SEM). Simvastatin treatment (weeks 4 to 6; Simva) of animals on normal diet (black bars) caused a significant decline in NogoA+ mature OLGs in the CC. Cuprizone-induced demyelination (gray bars) was associated with a decrease in NogoA+ mature OLGs (week 6) relative to animals on normal diet. Simvastatin treatment during the period of initial OPC proliferation/maturation (weeks 4 to 6) caused a significant reduction in NogoA+ cells relative to control. Although numbers of NogoA+ cells were maintained over time in vehicle treated animals on normal diet (from weeks 6 to 9), animals treated with simvastatin had a persistent decrease in NogoA+ OLGs in the CC by week 9. The cuprizone-vehicle treatment group demonstrated a recovery of NogoA+ OLGs at week 9 relative to week 6. However, simvastatin treatment during weeks 4 to 9 or 7 to 9 caused a significant decrease in mature OLGs in the CC. Analysis of variance *P* values <0.001. ns *P* > 0.05, **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

bel OLGs in human and mouse adult CNS to the same extent as other markers such as adenomatous polyposis coli, 2',3'-cyclic nucleotide 3'-phosphodiesterase, and proteolipid protein.^{15,25} NogoA+ mature OLGs were typically aligned in rows (Figure 5A).

Simvastatin Treatment Decreases Numbers of Mature OLGs under Non-Demyelinating Conditions

Animals on normal diet demonstrated maintenance of NogoA+ cell numbers over time (Figure 5B, Figure 6, A and B); there was no significant difference between OLG cell numbers from animals sacrificed at week 6 relative to week 9 (*P* > 0.05). The previously mentioned increase in Olig2^{strong} OPCs in the CC of these animals may reflect an ongoing replacement of mature OLGs observed in adult rodent CNS.^{23,24} Simvastatin treatment of animals on normal diet (weeks

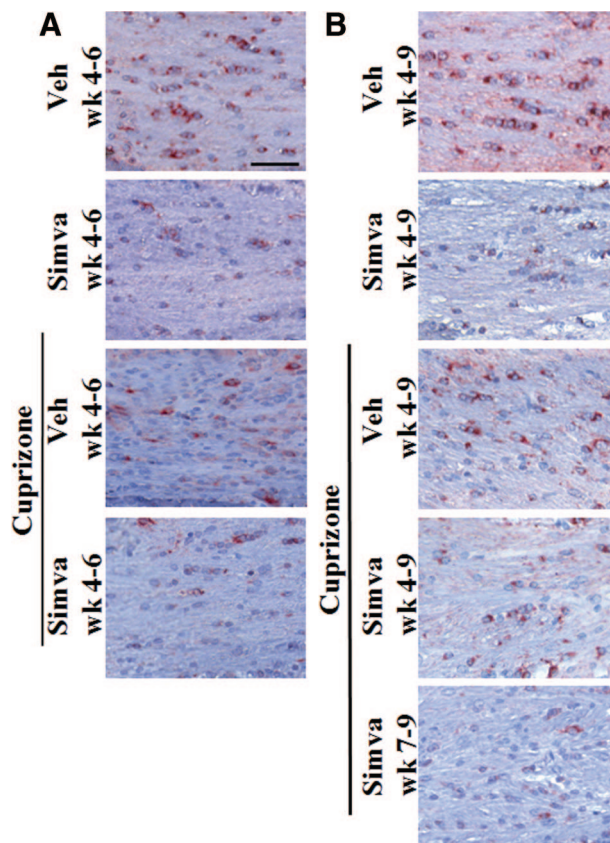


Figure 6. Simvastatin inhibits the recovery of mature OLGs in the demyelinated corpus callosum. **A:** Coronal sections of corpus callosum of animals sacrificed at week 6. Mature OLG cell bodies were labeled against NogoA (brown) and counterstained with hematoxylin (blue). Simvastatin treatment (weeks 4 to 6) of animals on a normal diet caused a significant decline in NogoA+ mature OLGs. Cuprizone treatment was also associated with a decrease in NogoA+ mature OLGs relative to animals on normal diet. Simvastatin treatment during the period of initial OPC proliferation/maturation (weeks 4 to 6) caused a further reduction in NogoA+ cells. **B:** Coronal sections of corpus callosum of animals sacrificed at week 9. Animals on a normal diet treated with simvastatin demonstrated a decrease in NogoA+ OLGs relative to vehicle control. Simvastatin treatment during weeks 4 to 9 or 7 to 9 caused a significant decrease in mature OLGs relative to cuprizone-vehicle controls. Scale bar = 50 μ m.

4–6, 4–9) caused a decrease in numbers of NogoA+ cells (Figure 5B, Figure 6, A and B) relative to the vehicle control, which is consistent with the reduced myelin load observed with long-term treatment, and with the conclusion that progenitor differentiation into mature OLGs was impaired in this condition.

Simvastatin Treatment Further Decreases Numbers of Mature OLGs during the Period of Concomitant Demyelination and Progenitor Responses

Cuprizone caused an expected decrease in NogoA+ cells at week 6, reflecting targeting of these cells by the toxin (Figure 5B, Figure 6A). Short-term statin treatment (weeks 4–6) of animals on cuprizone caused a further decrease in NogoA+ mature OLGs relative to cuprizone-vehicle controls (Figure 5B, Figure 6A), either reflecting impaired progenitor differentiation into mature OLGs or a direct cytotoxic effect on the OLGs.

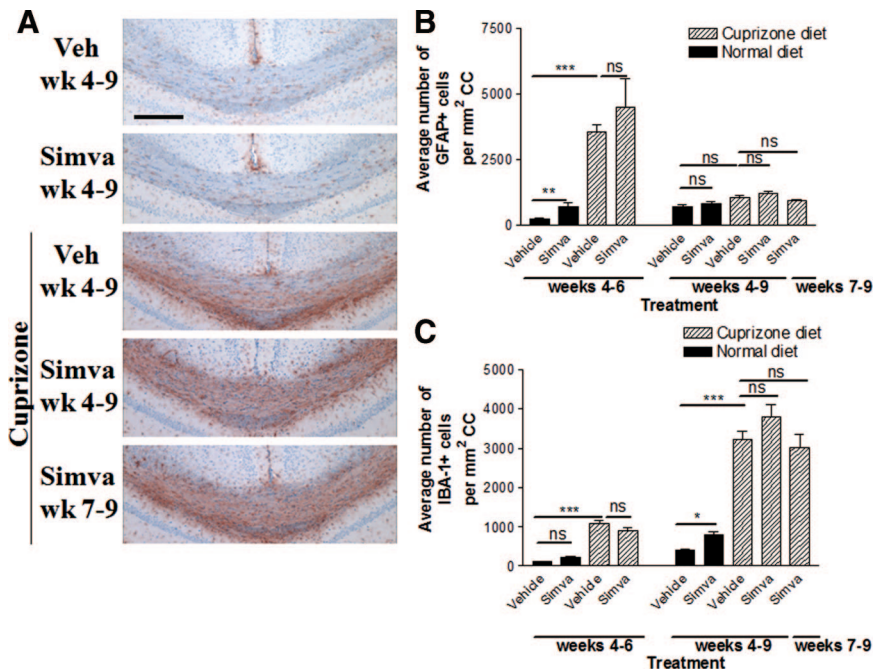


Figure 7. Simvastatin does not decrease gliosis in the cuprizone model. **A:** Representative images of sections immunostained against GFAP (astrocytes; brown) and counterstained with hematoxylin (blue). Scale bar = 200 μ m. **B:** Average numbers of GFAP+ astrocytes per mm² of the corpus callosum (CC) \pm SEM. Simvastatin treatment (weeks 4 to 6) of animals on normal diet caused an increase in astrocyte numbers relative to vehicle alone (black bars). Cuprizone administration (gray bars) increased astrocyte reactivity in the CC, which was not affected by concomitant simvastatin treatment. Long-term simvastatin treatment (weeks 4 to 9) of animals on normal diet showed no difference in astrocyte reactivity relative to vehicle alone. The cuprizone treated animals showed a decrease in astrocyte numbers by week 9 relative to week 6; similar profiles were observed when simvastatin was administered to cuprizone-fed animals. **C:** Average numbers of IBA-1+ microglia/macrophages per mm² of the CC \pm SEM. Simvastatin treatment (weeks 4 to 6) of animals on normal diet (black bars) had no significant effect on IBA-1+ cell number in the CC. By week 6, cuprizone administration (gray bars) caused an increase in IBA-1+ cell numbers relative to vehicle controls; this was not affected when simvastatin was administered. Long-term simvastatin treatment (weeks 4 to 9) of animals on normal diet was associated with an increase in microglia reactivity relative to control. At the end of the recovery from cuprizone-induced demyelination, microglia numbers were further increased relative to vehicle control. Simvastatin treatment of cuprizone-fed animals during weeks 4 to 9 or 7 to 9 had no effect on IBA-1+ cell numbers relative to cuprizone controls. Analysis of variance *P* values <0.001. ns *P* > 0.05, **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

Simvastatin Treatment Further Decreases Numbers of Mature OLGs during the Post-Cuprizone Remyelination Phase

By the end of the post-cuprizone recovery phase (weeks 7–9), the numbers of NogoA+ OLGs had recovered to values comparable with those observed in animals on normal diet for the entire duration of the experiment (*P* > 0.05, Figure 5B, Figure 6B). In comparison, simvastatin treated animals (weeks 4–9, 7–9) had significantly fewer NogoA+ cells relative to cuprizone-vehicle controls (Figure 5B, Figure 6B), supporting the conclusion that simvastatin blocked the differentiation of progenitors into mature OLGs.

Effects of Simvastatin Treatment on Astrocytes and Microglia in the Corpus Callosum

Although the cuprizone model has been demonstrated to not have any lymphocyte infiltration or blood-brain barrier breakdown,¹¹ there is significant gliosis in response to OLG injury and demyelination.²⁶ We assessed the potential confounding impact of simvastatin on glial reactivity in the CNS by measuring numbers of infiltrating microglia and astrocytes in the demyelinated CC following cuprizone treatment. We used IBA-1 and GFAP as markers for activated microglia and astrocytes, respectively, given the functional demonstration that these markers are up-regulated on glial activation.^{27,28}

Interestingly, following statin treatment of animals on a normal diet, there was an increase in numbers of activated astrocytes (week 6) and microglia (week 9) relative to vehicle control (Figure 7, B and C, respectively), suggesting a glial response to statin-induced damage to the CC.

At 6 weeks of cuprizone administration, we observed a significant increase in numbers of activated astrocytes (GFAP+; Figure 7, A and B) and microglia (IBA-1+; Figure 7C) in the CC relative to animals on normal diet. Simvastatin treatment of cuprizone-treated animals did not significantly decrease gliosis relative to the respective control (Figure 7, A–C). These results indicate that simvastatin does not dampen glial activation responses in the brain of cuprizone-treated mice, and that any statin-induced neural response during remyelination is not confounded by effects on glial reactivity.

Discussion

Using immunomodulatory concentrations, our study indicates that long-term simvastatin therapy exerts effects on myelin and oligodendroglial cells in normal, demyelinating, and remyelinating environments. We demonstrate that simvastatin interferes with myelin repair and maintenance by directly impacting OPC functions and affecting mature OLG numbers. Klopffleisch et al. (2008) have now also shown that

treatment with statin in a range of doses also reduces myelin content in cuprizone-treated animals.²⁹

Long-Term Simvastatin Treatment Interferes with Myelin Maintenance under Non-Demyelinating Conditions

Short-term simvastatin therapy alone did not induce any notable effects on myelin in the CC of animals on normal diet, consistent with previous studies showing that lovastatin treatment (3 weeks) had no effect on myelin protein and mRNA levels in the mouse spinal cord.⁸ However, we observed that long-term simvastatin treatment of animals on normal diet (6 weeks) resulted in a reduction in myelin load and a loss of mature OLGs in the CC. Chronic simvastatin treatment has been shown to significantly reduce brain cholesterol levels in healthy mice, whereas lovastatin does not.¹⁹

Mature oligodendrocytes are the primary producers of cholesterol in the brain.³⁰ Cholesterol is highly concentrated in oligodendroglial membranes and undergoes a continuous turnover in myelin.³¹ Cholesterol is also concentrated in fluid microdomains in the membrane bilayer, termed lipid rafts, which house and aggregate signaling molecules to facilitate the initialization of intracellular signaling cascades. Lipid rafts in oligodendrocytes are also significantly distinct from those in other cell types; the aggregation of these lipid rafts forms the myelin sheath. Abnormal myelination has previously been observed when cholesterol is depleted in mouse organotypic spinal cord cultures³² or by genetic manipulation of OLGs *in vivo*.³⁰ Treatment of rat OLGs with lovastatin *in vitro* inhibits cholesterol-dependent transport of proteolipid protein mRNA into oligodendroglial membrane processes, resulting in the formation of abnormal myelin-like sheaths devoid of this myelin protein.³³ Treatment of mouse organotypic or human explant cultures with cholesterol biosynthesis inhibitors such as statins for prolonged periods of time causes cell death of oligodendrocytes.^{32,34,35} In addition, simvastatin may influence oligodendroglial cell survival signaling via blockade of isoprenylation.³⁶ In this regard, treatment of fully myelinated mouse cerebellar slices with both nanomolar and micromolar doses of simvastatin for 6 days has been shown to induce significant OLG cell death, which is fully rescued by supplementation with the isoprenoid farnesyl pyrophosphate.³⁵

Increased numbers of both Olig2^{strong} and Nkx2.2^{strong} OPCs in simvastatin-treated animals on normal diet likely reflects a block in differentiation at the pre-OLG state. These OPCs were not contributing to myelin formation and maintenance, as evidenced by reduced myelin load in the CC, and the loss of mature OLGs. OPC differentiation is associated with the acquisition of distinct sets of lipid rafts housing signaling molecules relevant for the maturation process.³⁷ Indeed, the recruitment of cytoskeletal-associated proteins to lipid rafts in oligodendrocytes is important for the initiation of myelination. Statin treatment has been associated with a decrease in levels of membrane cholesterol and of a lipid raft marker, flotillin, demonstrating the ability of statin-induced cho-

lesterol depletion to disrupt lipid rafts.³⁸ The increase in gliosis that we observed in the CC of these animals suggests that simvastatin-induced injury to the CC could also have promoted the recruitment of OPCs. The increase in OPCs in the CC of simvastatin-treated animals is unlikely to reflect an increase in proliferation given that *in vivo* statin treatment has been previously shown not to induce changes in mRNA levels of genes implicated in OPC proliferation, and that statins inhibit human adult progenitor proliferation *in vitro*.^{8,10}

Simvastatin Treatment Inhibits Initial OPC Responses to Demyelination and Impairs Subsequent Remyelination

Simvastatin treatment inhibited both the initial OPC responses observed during cuprizone-induced demyelination, and myelin repair following return to normal diet. These results likely reflect simvastatin's inhibition of the new cholesterol synthesis that is deemed important for remyelination in the cuprizone model.²⁶ Simvastatin treatment may have interfered with myelin repair by inhibiting lipid-raft associated signaling and cholesterol-dependent process extension.³⁹

The brain synthesizes its own source of cholesterol from fetal development throughout adulthood, which cannot be supplemented by dietary or circulating cholesterol derived from the liver.^{30,40} Increases in cholesterol and the brain-specific cholesterol metabolite 24-S-hydroxycholesterol have been measured in patients with active MS and in animals in the active phase of experimental autoimmune encephalomyelitis.^{40,41} This likely either reflects membrane cholesterol found in debris resulting from neuronal and oligodendroglial injury and death, or the increase in cholesterol synthesis proposed to be necessary for repair. The CSF of primary progressive or patients with long-time relapse-remitting MS has reduced levels of 24-S-hydroxycholesterol relative to healthy controls⁴¹; this may reflect cell loss and consequent reduction in cholesterol production, and may suggest that repair processes may be further impaired by simvastatin.

Simvastatin treatment during the period of concomitant OPC proliferation/maturation and demyelination caused a decrease in both Olig2^{strong} and Nkx2.2^{strong} OPCs. Exposure to simvastatin *in vitro* can hinder progenitor cell migration, inhibit proliferation, and exert a cytotoxic effect.^{9,10} Previous studies in the experimental autoimmune encephalomyelitis model showed that lovastatin treatment (3 weeks) enhanced OPC proliferation, differentiation, and recruitment to the spinal cord.⁸ Such statin therapy significantly inhibits the immune response within the experimental autoimmune encephalomyelitis-afflicted CNS,⁶ raising the issue as to whether the net observed effects are indirectly mediated through anti-inflammatory effects. Furthermore, when either lovastatin or simvastatin are chronically administered daily to mice, simvastatin is found at higher concentrations in the CNS, induces changes in expression in a higher number of genes, and significantly reduces brain cholesterol levels, relative to lovastatin.¹⁹ Our data indicate that prolonged simvastatin

treatment impeded remyelination on return to normal diet by maintaining OPCs in an immature state (Olig2^{strong}/Nkx2.2^{weak}), thereby preventing maturation into NogoA+ OLGs that contribute to formation of new myelin. The observed increase in Olig2^{strong} OPCs in the CC at this time is unlikely to reflect enhanced migration or proliferation given that simvastatin inhibits rodent OPC migration and human progenitor proliferation *in vitro*.^{9,10} Our postulate is supported by the finding that OPC differentiation requires the acquisition of distinct sets of cholesterol-enriched lipid rafts.³⁷ We also observed that the number of Nkx2.2^{strong} cells were significantly more reduced when simvastatin was administered to cuprizone-fed animals for weeks 4 to 9 in comparison with weeks 7 to 9, thereby indicating that prolonged exposure to the drug may be associated with more pronounced effects on these cells. Nkx2.2 expression is increased before terminal oligodendrocyte differentiation.^{15,22} The loss of mature OLGs when simvastatin was administered during the demyelination or remyelination phases is supported by cytotoxic effects of statins on mature OLGs *in vitro*,^{9,34,35} but may also reflect the lack of maturation of progenitors under these treatment regimens.

Conclusion

Together, our data support the conclusion that simvastatin blocks the differentiation of progenitors into mature myelinating cells, thereby inhibiting remyelination in the cuprizone model. We also show the importance of cholesterol and isoprenoid synthesis pathways in processes of myelin maintenance and remyelination. Our findings also highlight the necessity of monitoring long-term effects of systemically applied therapies that can access the CNS, particularly those that can impact cell types that are postulated to be targeted in neurological disease processes and that are implicated in any tissue repair process. The expression of the enzyme inhibited by statins, HMG Co-A reductase, in all cell types along with the penetration of the lipophilic simvastatin into the brain parenchyma, together suggest potential direct effects of statins on neural cell properties.

Acknowledgments

The authors thank Rozica Jeneca Bolovan for tissue processing and immunohistochemistry.

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