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Neurobiology

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, Münster, 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 Olig2strong and Nkx2.2strong 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 Nkx2.2strong OPCs and an increase in Olig2strong cells, suggesting that OPCs were maintained in an immature state (Olig2strong/Nkx2.2weak). 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

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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.\textsuperscript{10} Prolonged exposure to statins, however, can cause oligodendroglial process retraction through cholesterol depletion, and cell death by blocking synthesis of isoprenoids and cholesterol.\textsuperscript{9}

Given the multiple \textit{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 \textit{in vivo}. In our current study we used oral cuprizone administration as an \textit{in vivo} model of non-immune system-initiated, brain-localized demyelination and remyelination.\textsuperscript{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.\textsuperscript{13} The concomitant demyelination and OPC responses/remyelination observed with cuprizone administration replicates what has been observed in demyelinating MS lesions.\textsuperscript{14} This model also provides the opportunity to study remyelination alone once the cuprizone toxin is removed from the diet.\textsuperscript{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.\textsuperscript{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.\textsuperscript{16–18}

We elected to use simvastatin due to its use in MS clinical trials,\textsuperscript{7} its ability to cross the blood-brain barrier,\textsuperscript{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.\textsuperscript{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.

\textbf{Materials and Methods}

\textit{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 \textit{ad libitum} (bis-cyclohexane oxalidihydrazone; 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 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).\textsuperscript{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.\textsuperscript{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.

\textit{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

![Figure 1. Treatment protocol. Cuprizone was administered for weeks 1 to 6 to induce demyelination in the corpus callosum (CC). A: OPC proliferation/remyelination 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.]
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 coun-
terstained with H&E and subsequently dehydrated. Im-
munohistochemistry 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, Can-
da) at elevated pressure. All samples were treated for
endogenous peroxidase activity with 0.3% H₂O₂ followed
by blocking with LVBlock (LabVision). Sections were in-
cubated with the appropriate antibodies: mouse anti-
myelin basic protein (MBP; Stemmermonoclonals, Lutherville, MD), rabbit anti-glial fibrillary acidic protein
(GFAP; Medcorp, 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 pro-
tein A (NogoA; Chemicon, Temecula, CA). Primary anti-
bodies were detected using avidin-biotin (LabVision), LV
polymer (LabVision), or EnVision (Dako) amplification,
followed by visualization with horseradish peroxidase-cata-
yzed 3-amino-9-ethylcarbazole (LabVision) chromo-
gen deposition. Control sections showed low non-
specific staining. All sections were blindly digitized using a
Nikon Eclipse 55i microscope (Nikon Canada, Missis-
sauga, ON, Canada) equipped with a QICAM 12-bit Fast
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 choles-
terol 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 centrif-
gufed. Solvent was evaporated overnight, and pellets of
concentrated cholesterol were re-dissolved in reaction
buffer. Cholesterol levels were assessed using the Am-
plex 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 sec-
tions 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 histo-
chemical stains.¹² The area of MBP immunohistochemi-
cal staining was assessed objectively using an optical
density image (Scion Image software) calibrated to ×20
objective image. OPCs (Oligo⁺, Nkx2.2⁺, OLGs
(NogoA+), astrocytes (GFAP+), and microglia (IBA-1+)
were blindly counted in the medial corpus callosum in
×10 to ×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 mm²/area of MBP staining (μm²) ± SEM. All
comparisons between conditions were performed using
one-way analysis of variance followed by Newman-Keuls
multiple comparison posthoc test. Normality was as-
essed using Bartlett’s test for equal variances. Probabil-
ity 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 immunomodula-
tory directed studies.⁶,⁸ Statin was confirmed to be func-
tionally 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 μg/ml) relative to vehicle-treated con-
trol (139.8 μg/ml). This was also noted when simvastatin
was administered to cuprizone-fed animals (weeks 4 to 9,
17.8 μg/ml; weeks 7 to 9, 34.1 μg/ml) as compared with
cuprizone alone (86.7 μg/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 determin-
ing the area of staining of MBP in digitized immunohisto-
chemical 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-de-
myelinating 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 (Fig-
ure 2B). However, long-term simvastatin therapy alone
given to animals on normal diet (weeks 4 to 9) did signif-
ificantly 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.
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 determining 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
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,\textsuperscript{15,21,22} and determined the average number of strongly positive cells in the medial CC. In the normal CC, both Olig2\textsuperscript{strong} and Nkx2.2\textsuperscript{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\textsuperscript{strong} cells (\(P < 0.01\), Figure 3C, Figure 4, A and B) and a significant decrease in Nkx2.2\textsuperscript{strong} cells (\(P < 0.001\), Figure 3D, Figure 4, A and B) at week 9 as compared with week 6. Since Olig2\textsuperscript{strong}/Nkx2.2\textsuperscript{weak} cells are regarded as immature OPCs\textsuperscript{22}, 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.\textsuperscript{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\textsuperscript{strong} (Figure 3C, Figure 4, A and B) and Nkx2.2\textsuperscript{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\textsuperscript{strong} and Nkx2.2\textsuperscript{strong}).\textsuperscript{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\textsuperscript{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.\textsuperscript{21} This increase was not associated with a simultaneous increase in Nkx2.2\textsuperscript{strong} cells (Figure 3D, Figure 4A). There was a strong correlation between demyelination score and average numbers of Olig2\textsuperscript{strong} cells in the CC (\(R^2 = 0.82\)), and a weaker correlation with numbers of Nkx2.2\textsuperscript{strong} cells (\(R^2 = 0.66\)) at the end of the demyelination period (week 6). Our results suggest that immature OPCs (Olig2\textsuperscript{strong}/Nkx2.2\textsuperscript{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 numbers of Nkx2.2\textsuperscript{strong} OPCs in the CC relative to cuprizone alone. Analysis of variance \(P\) values <0.001. ns \(P > 0.05\), \(* P < 0.05\), \(* * P < 0.01\), \(* * * P < 0.001\).

**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-
zone treatment showed a trend toward a decrease in Olig2[^2] 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[^2] 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[^2] cells. The more prolonged simvastatin treatment regimen (weeks 4 to 9) also induced a significant decrease in Nkx2.2[^2] 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[^2] OPCs into the CC such that they were only increased by week 9, that continued myelin injury recruited more Olig2[^2] OPCs to the CC, or that OPCs were maintained in an immature state (Olig2[^2]/Nkx2.2[^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-
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. 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 mature OLGs 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 Olig2strong OPCs in the CC of these animals may reflect an ongoing replacement of mature OLGs observed in adult rodent CNS. Simvastatin treatment of animals on normal diet (weeks 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 mature OLGs 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.
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, A and B), 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-fed animals during weeks 4 to 9 or 7 to 9 had no effect on IBA-1+ cell numbers relative to vehicle control. Analysis of variance $P$ values $<0.001$. ns $P > 0.05$, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

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. Klopfleisch 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 Olig2strong and Nkx2.2strong 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 cholesterol 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.30 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 controls31; 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 Olig2strong and Nkx2.2strong 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 (Olig2strong/ Nkx2.2weak), thereby preventing maturation into NogoA+ OLGs that contribute to formation of new myelin. The observed increase in Olig2strong 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 Nkx2.2strong 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 cytokotic 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.

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