Oligodendrocyte progenitors and large volume demyelination

Delayed Oligodendrocyte Progenitor Cell Recruitment and Maturation in Large Volume Demyelination

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Oligodendrocyte progenitors and large volume demyelination

Abstract

Remyelination-inducing therapies have the potential to restore neurologic function and prevent progression in multiple sclerosis (MS). However, preclinical therapies capable of driving oligodendrocyte differentiation and enhancing remyelination in murine models have not yet yielded meaningful clinical improvement. These setbacks suggest that small animal models may be inadequate for the development and identification of promising regenerative therapies. Unlike MS, rodent models of demyelination undergo highly efficient remyelination, have typically very small volumes and exhibit abundant oligodendrocyte recruitment rapidly following demyelination. We hypothesized that oligodendrocyte progenitor cell (OPC) recruitment becomes rate-limiting as lesion volume increases leading to a failure of oligodendrocyte differentiation and endogenous remyelination. In this study, we utilized lysolecithin injection into the large white matter tracts of the rabbit to create demyelinated lesions that have cross-sectional areas 20-fold larger than those previously reported in mice, and volumes 8-fold larger. Unlike rodent lesions which show robust supernumerary OPC recruitment (representing a 3-10 fold increase in Olig2⁺ cell density), recruitment in the rabbit never exceeds normal levels. Importantly, the density of mature CC1⁺ oligodendrocytes failed to return to NAWM levels by 56 dpl and the percentage of differentiated oligodendrocytes drastically lagged behind that of rodent lesions. These results suggest that as lesion volume increases the requirement for increased OPC proliferation and migration becomes limiting. As oligodendrocyte differentiation from human OPCs is dependent on the local density of progenitors, the reduced density of progenitors in large lesions may contribute to failed differentiation and remyelination. Together, we show that large volume demyelination in the white matter of the rabbit recapitulates several key features of human MS lesions, and represents an important preclinical model for the assessment of remyelination therapies in human demyelinating disease.

Oligodendrocyte progenitors and large volume demyelination

INTRODUCTION

Primary demyelination is most commonly associated with multiple sclerosis (MS), where half of patients will require walking assistance after a decade of disease duration¹. Immune-mediated demyelination and loss of oligodendrocytes (OL) results in loss of neurological function. In experimental models, remyelination relies on restoration of the OL population through the recruitment (both migration and proliferation) and subsequent differentiation and maturation of oligodendrocyte precursor cells (OPCs)². OPCs are a multipotent stem cell population that are distributed throughout the adult CNS. While the role of adult OPCs in normal brain remains elusive, OPCs are responsible for the replacement of lost OLs in the context of demyelination³. Following a demyelinating event, OPCs migrate into the damaged region from adjacent healthy tissue where they become activated⁴ and begin to proliferate. The expression of activation markers, such as Sox2, are downregulated as OPCs begin to differentiate and mature⁴. Newly generated OLs engage and ensheath denuded axons and upregulate myelin proteins resulting in the production of a thin myelin sheath with shorter internodes that is capable of restoring saltatory conduction and neurological function⁵.

While remyelination is a common feature of early relapsing-remitting MS, remyelination failure becomes more common with increasing disease duration and age. Remyelination failure is thought to be due to the malfunction of one or more stages of oligodendrocyte regeneration: namely OPC recruitment, differentiation, and myelin formation². While quiescent OPCs are associated with chronic demyelination in MS⁶, the prevailing theory is that remyelination fails in MS due to a failure of differentiation. In response, a number of drug candidates aimed at boosting endogenous remyelination through the promotion of OPC differentiation have been developed and advanced to clinical trials after successful preclinical results, notably opicinumab (anti-Lingo1) and clemastine⁷⁻⁹. Unfortunately, while both were able to significantly improve visual evoked potential latency indicative of remyelination in the optic nerve, neither was able to significantly impact clinical outcome or imaging endpoints in initial trials¹⁰⁻¹². The paradoxical success of differentiation-promoting therapies in rodent models, but little success in clinical trials suggests fundamental differences in the rate-limiting steps between remyelination in animal models and human MS.

In rodent models of demyelination, there is little evidence that the rate of OPC recruitment is rate-limiting. For example, when the number of OPCs within the lesion is experimentally increased via overexpression of PDGF-A there is no change in the time course or extent of remyelination¹³. Additionally, relatively few rounds of division are required to restore OPC density in rodent lesions (1-3 cycles)¹⁴, and when nearly all OPCs are pharmacogenetically depleted in

Oligodendrocyte progenitors and large volume demyelination

the rodent brain OPC densities are restored in less than 10 days¹⁵. Indeed, it is only after long-term administration of cuprizone to chronically deplete of OPCs over the period of several months that recruitment fails and remyelination is inefficient¹⁶. As OPCs are recruited from the adjacent NAWM, the burden of both migration and proliferation disproportionately increases with increased lesion volume. In MS, individual demyelinating lesions have volumes of several thousand cubic millimeters¹⁷. In contrast, the volume of focal demyelinated lesions modelled in rodents is very small, typically less than 1 mm³^{18,19}. Thus, we hypothesized that OPC recruitment becomes rate-limiting as lesion volume increases leading to a failure of OL differentiation and endogenous remyelination.

In this study we sought to develop a model that more accurately recapitulates the burden of recruitment seen in MS lesions. To do this we utilized the large white matter tracks of the rabbit to create demyelinating lesions with cross-sectional areas 20 times that produced in mice. Along with increased lesion size, OPC recruitment was substantially inferior to corresponding rodent models and closely resembled the pattern observed in MS. Large volume rabbit lesions exhibited distinct regional differences in the pattern of gliotic response, axonal injury, OPC recruitment, activation and proliferation that evolved with time following induction of demyelination. Together, we describe lysolecithin-induced lesions in the rabbit that recapitulate several key features of demyelination in MS which may more accurately predict the outcome of novel remyelination-inducing therapies in clinical trials.

Oligodendrocyte progenitors and large volume demyelination

MATERIALS AND METHODS

Animals and surgery

All experiments were performed according to protocols approved by the University at Buffalo's Institutional Animal Care and Use Committee. New Zealand White Rabbits were purchased as needed (Female, average weight 2.96 ± 0.13 kg and average age 15.79 ± 0.53 weeks) (Charles Rivers Laboratories, Wilmington, MA). Induction anesthesia was accomplished via intramuscular injection of ketamine (35 mg/kg) and xylazine (5 mg/kg) into epaxial muscles. After loss of consciousness, supplemental heating began and corneas were lubricated. The proximal 3 cm of the tail was shaved to provide an optimal location for the pulse oximetry probe (far preferable to use of ear or tongue). ECG leads and rectal temperature probe were placed. After pre-oxygenation via face mask, rabbits were intubated in ventral recumbency with heads and necks held in extension. A size 3.0 endotracheal tube was threaded over an 8-French urinary catheter. After placement of the catheter through the glottis, the ET tube was advanced over the catheter and into the trachea. Successful intubation was verified via immediate capnography. Upon success, isoflurane was delivered via Bain Circuit (1-3%). An IV catheter was placed (marginal ear vein), and the surgical site was shaved and prepped prior to moving the rabbit to the operating room (OR). Pre-emptive, initial analgesics were administered (0.05 mg/kg buprenorphine, subcutaneously (SC) and 1.5 mg/kg carprofen, SC). Once moved onto the OR table, rabbits were immediately reconnected to isoflurane to ensure a suitable anesthetic plane. Then, they were transiently disconnected from the Bain Circuit to allow loose movement of the head into the stereotaxic frame (Kopf Model 902 Small Animal Stereotaxic Instrument + Kopf Model 1240 Rabbit Adaptor + Rabbit Risers, Kopf Instruments, Tujunga, CA). The endotracheal (ET) tube, was carefully moved to rest between the arms of the Kopf Rabbit Adaptor. It was important that the ET tube was not advanced beyond 12 cm to avoid bronchial obstruction. Once positioned, the capnograph and Bain Circuit were quickly reconnected. At this time further careful positioning of the rabbit's head was possible via tooth bar, zygoma clamps and nose clamp. General anesthesia could be maintained for the remainder of the stereotaxic procedure with isoflurane. Spontaneous respirations were augmented by manual intermittent positive pressure ventilations (IPPV). 0.1 mL bupivacaine (0.25%) or lidocaine (2%, diluted 1:1 with 0.9% NaCl) was injected SC. A 7-8 cm incision was made along the midline of the scalp to expose both bregma (intersection of the sagittal and coronal sutures) and lambda (intersection of the sagittal and lambdoid sutures). Both left and right arm Hamilton needles were filled with distilled H₂O to prevent occlusion and ensure flow prior to lysolecithin loading. Placing the needles point on either bregma or lambda, the dorsal-ventral (DV) relationship was adjusted using the

Oligodendrocyte progenitors and large volume demyelination

Kopf Rabbit Adaptor until bregma was 1.5 mm above lambda. The arms of the Kopf frame were adjusted to a 20° angle, and the needle points were placed on bregma. The anterior-posterior (AP), lateral-medial (LM), and DV of each arm was recorded. The following adjustments were made to these values: AP= +0.5 mm, LM= +7.7 mm, DV= -5.7 mm. Injection site was confirmed via Evans Blue injection (**Supplemental Figure 3A**). Drill bits (Burs for Micro Drill 19007-29, 2.9 mm, Foster City, CA) were sterilized via dry micro bead bench side sterilizer for 1 minute and cooled with room temp saline. In advance of drilling, topical articaine (4%) was dripped onto the skull and allowed to absorb and dry over drilling skull sites. Drill holes were made through the skull to the level of the meninges with constant saline stream to prevent parenchymal heat damage. A 30G needle was then used to puncture the meninges to allow for free passage of the Hamilton needle. 1% lysolecithin was then injected at a rate 0.1 µl/min, with 5 minute wait times between microliters to allow for diffusion. Once injection was completed, the needles were left in place for 20 additional minutes to allow for diffusion.

Post-operatively, buprenorphine was administered (0.05 mg/kg, SC) 4 hours after the initial dose, and carprofen (1.5 mg/kg, SC) was continued twice daily for a total of 4 consecutive days. Rabbits were monitored daily after surgery and assessed for: mentation, weight loss from baseline, appetite, water consumption, amount and character of feces, amount of urination, and presence of neurologic signs. Post-surgical weight loss peaked at 28 dpl, followed by a rapid recovery (**Supplemental Figure 3B-C**).

Tissue processing

Animals were sacrificed at 7, 14, 21, and 56 days post-lesion (dpl) by transcardial perfusion of saline followed by 4% paraformaldehyde under deep anesthesia. Following decapitation, whole brain tissue was extracted and post-fixed for 30 minutes in 4% paraformaldehyde. For cryopreservation, the tissue was first left in 1x PBS overnight, then transferred into a 7.5% sucrose solution overnight, and finally 15% sucrose overnight. Cryoprotected tissue was then frozen in optimal cutting temperature medium (Tissue-Tek). Serial, 16 µm-thick coronal sections were cut using a Leica cryostat, and stored in -80 °C freezer until processing.

To identify lesion location and volume, every 10th section was washed with 1x PBST (x3 by 5 min), stained with FloroMyelin for 1 hour at room temperature (1:300, Thermo Fisher Scientific) and 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) for 3 minutes (1:5000, Sigma Aldrich), and mounted in prolong gold (Thermo Fisher Scientific). Lesions were identified as regions with lesser FloroMyelin signal and corresponding hypercellularity by DAPI, compared

Oligodendrocyte progenitors and large volume demyelination

to surrounding white matter. Cross-sectional lesion areas were calculated for both left and right hemisphere lesions using the NIH ImageJ software, and the largest cross-sectional area for each lesion was identified as the lesion center.

Estimation of lesion volume

Lesion volume was calculated from the measured cross-sectional areas determined by FloroMyelin staining using Cavalieri's estimator of morphometric volume⁵⁶:

$$V_c = d \left(\sum_{i=0}^n (y_i) \right) - (t)y_{MAX}$$

Where V_c is the Cavalieri's estimated volume, d is the distance between sections, y_i is the cross-sectional area of section i , t is the thickness of the sections, and y_{MAX} is the largest cross-sectional area of the lesion. Since every 10th section was stained, $d = 160 \mu\text{m}$, and $t = 16 \mu\text{m}$.

Immunohistochemistry (IHC)

Slides immediately adjacent to the lesion centers, identified by FloroMyelin staining, were used for all immunohistochemical procedures. In general, sections were permeabilized for 1 hour with 1% Triton X-100 (Alfa Aesar) and 0.25% Tween 20 (Calbiochem). Sections were blocked for 1 hour with a solution containing 0.5% Triton X-100, 5% bovine serum albumin (BSA), and 5% normal donkey serum. Primary antibodies utilized were goat anti-Olig2 (1:100, R&D Systems), mouse anti-CC1 (1:50, Millipore), mouse IgG2a anti-SOX2 (1:100, R&D Systems), mouse IgG1 anti-Ki67 (1:25, Fisher Scientific), rat IgG2a anti-MBP (1:300, Abcam Inc.), goat IgG Smi311 & Smi312 (1:1000, BioLegend), rabbit IgG anti-App (1:500, Thermo Fisher), rabbit IgG anti-Iba1 (1:300, Wako Chemicals), and mouse IgG1 anti-Gfap (1:400, Sigma-Aldrich). Alexa 488, 594, and 647-conjugated secondary antibodies (Invitrogen) were used at 1:500. Lesion areas were captured at 10X and 20X magnification using an Olympus IX83 with IX3-SSU motorized stage and image tiles stitched via cellSens software. Images of immunostained sections were aligned with their corresponding FloroMyelin stained section to accurately mark lesion boundaries, with exclusion of any portion of the lesion which extends into the gray matter. Cells counts were determined via a deep learning (machine learning) augmented process (code available upon request), validated with multi-counter manual counts. The Keras based convolution network was trained on a set of ~3250 CC1 positive and ~3250 CC1 negative pre-classified images (supervised learning).

Oligodendrocyte progenitors and large volume demyelination

Statistics

All statistical testing was completed using GraphPad Prism Version 8.4.0 (455). The control group for all analyses of cell number and phenotype was NAWM. Groups comprised either time-points following injection of lysolecithin and/or regional analysis within the lesion. For all reporting, an experimental unit comprised a single lesion. Given the novelty of the method, sample size was based on previous experience with gliotoxin lesions established in mouse and rat. A minimum of 4 experimental units (lesions) per group was set *a priori* and along with a requirement for at least three animals per group. Criteria for lesion inclusion were established *a priori*. Only lesions located within internal capsule white matter were included in the analysis. No white matter lesions regardless of size were excluded from the analysis. Rabbits were allocated to each group in a randomized fashion based on availability from the vendor and surgical schedule. Manual cell counting for all quantitative measures was performed in a blinded fashion. Normality of data was tested in GraphPad using the D'Agostino-Pearson omnibus test. For analysis of time-series data, one-way ANOVA with Tukey's post-hoc testing was performed. For analysis of time-series and region delimited data, two-way ANOVA with Sidak's post-hoc testing was performed.

Data availability

The data that support the findings of this study are available upon reasonable request.

RESULTS

Abundant OPC recruitment is universal in rodent models but is relatively uncommon in MS lesions.

To compare OPC recruitment between experimental models and human disease, we performed a meta-analysis of absolute and relative OPC densities following demyelination. While the recruitment of OPCs occurs following demyelination in MS, the density of OPCs found within lesions does not exceed that of normal appearing white matter (NAWM) (**Supplemental Figure 1A**). In contrast, rodent models of toxin-induced demyelination are associated with supernumerary OPC recruitment and highly efficient remyelination^{20, 21} (**Supplemental Figure 1B**). Likewise, following inflammatory demyelination or viral-mediated encephalomyelitis with attendant axonal injury and immune cell responses, OPC recruitment is similarly robust (**Supplemental Figure 1C**). As such, we hypothesized that supernumerary densities of OPCs are necessary for efficient remyelination, and that MS lesion tissue displays relatively weak OPC recruitment because OPC recruitment becomes rate-limiting as lesion volume increases.

Lysolecithin-induced lesions in the rabbit were larger and more persistent than those created in smaller rodent models.

In order to test the hypothesis that lesion volume influences OPC recruitment we sought an experimental species which had white matter structures that would allow for the production of lesions significantly larger than those induced in mice and rats. We selected the rabbit brain as the accessible white matter volume was 6 to 14 times larger²² than current rodent models, and we predicted that lesions could be generated with cross-sectional areas between 10 and 40 times greater than rat and mouse brain (**Fig. 1A**, ovals). To induce demyelination, we stereotactically injected lysolecithin into the internal capsules of adult NZW rabbits (bilateral injections of 1% lysolecithin, 5 µl). We first examined lesion formation at 14 days post lesion (dpl). Similar to murine lesions, lysolecithin induced profound focal demyelination at the site of injection and a clearly demarcated region of hypercellularity that precisely matched the region of myelin loss (**Fig. 1B**, white dashed line). Volumetric analysis by serial section reconstruction of the lesion indicated a comet-like appearance with a relatively spherical region of demyelination corresponding to the site of injection and a tapered region extending in the posterior-ventral direction, likely due to diffusion within the fascicles of the internal capsule. The mean cross-sectional area and volume peaked at 14 dpl (area: $2.21 \pm 0.3 \text{ mm}^2$, volume: $3.29 \pm 0.3 \text{ mm}^3$, $n = 8$) (**Fig. 1C-D**). Compared to mouse, rabbit lesions were over 8-fold larger in volume and a 20-fold increase in maximal cross-sectional area (**Fig. 1B-C**). Intriguingly, the 8-fold increased volume in rabbit exceeded the

Oligodendrocyte progenitors and large volume demyelination

5-fold increase in volume of lysolecithin injected. Following the peak in lesion area at 14 dpl, lesion area gradually declined with time (21 vs 14 days, Tukey's $p = 0.0280$). In stark contrast to both mouse and rat models, large regions of demyelination persisted in the rabbit even at 56 dpl by which time rodent models have typically completed remyelination (**Fig. 1E**).

Rabbit lesions represent areas of demyelination with preserved axons and prolonged gliotic response.

Next, we sought to characterize the extent of the gliotic response in these lesions, as well as determine the extent of axonal injury and loss in this new model. MBP staining showed abundant myelin debris at 7 dpl, with substantial clearance by 14 dpl (**Fig. 2A**). At 56 dpl, there was a partial recovery of MBP immunoreactivity, but lacked the structure observed in NAWM (**Fig. 2A**). Consistent with primary demyelination, a large number of neurofilament (NF)-labelled axons persisted following demyelination (7-56 dpl) (**Fig. 2B**). We observed a gradient of axonal density with greater numbers of axons at the lesion edge (**Fig. 2B**) (**Fig. 3**). Interestingly, low axonal densities in the lesion core is similarly observed in MS lesions²³. Additionally, axonal density did not decrease further beyond 7 dpl, based on a qualitative analysis (**Fig. 3**). Consistent with an initial injury, we observed evidence of axonal dysfunction by accumulation of App immunoreactivity at 7 dpl and a decline in the overall proportion of App⁺NF⁺ axons thereafter (**Fig. 2B**). Retraction bulbs, a common feature of MS lesion tissue¹⁷, were noted at all timepoints in and around the lesions (**Fig. 3**).

All lesions exhibited increased numbers of astrocytes (Gfap) and microglia/macrophages (Iba1), with the greatest number of microglia localized within the lesion core (**Fig. 2C-D**). Within the core, ameboid microglia commonly formed vesicular structures that were surrounded by Gfap⁺ astrocytic processes (**Supplemental Figure 2**). In addition to astrocytes and microglia, fibroblasts are recruited into mouse spinal cord lysolecithin and EAE lesions²⁴. However, we observed few Col1⁺ (fibroblast) cells within the lesion at 7 dpl and no Col1⁺ cells at 56 dpl (data not shown). Next, we quantified the overall gliotic (microglial and astrocytic) response by determining the density of nonoligodendrocyte lineage cell population (nonOligo, Olig2⁺DAPI⁺) within the lesion at each time point. The density of nonOligos increased 10-fold following injury, with densities peaking at 14 dpl (NAWM ($n = 20$) vs 14dpl ($n = 7$), Tukey's $p < 0.0001$) (**Fig. 2F**). While there was a statistically significant decrease in NonOligo cell density between 14 and 56 dpl (14dpl ($n = 7$) vs 56dpl ($n = 5$), Tukey's $p < 0.0001$), the percentage of nonOligos remained constant (14dpl ($n = 7$) vs 56dpl ($n = 5$), Tukey's $p = 0.64$) (**Fig 2F-G**). However, there was a statistically significant negative trend from 7dpl to 56dpl in the percentage of nonOligos (Test for linear trend, 7dpl to

Oligodendrocyte progenitors and large volume demyelination

56dpl, slope = -2.7, $F(1, 17) = 8.70$, $p = 0.009$). Together, this suggests that the gliotic response is persistent and remains active for at least the first two months following demyelination.

Demyelinated areas show insufficient OPC recruitment and failed to restore normal oligodendrocyte cell density.

As recruitment varies greatly between experimental models and MS lesions, we next sought to characterize OPC recruitment and differentiation following demyelination in this new model. We found that total oligodendrocyte lineage (Olig2⁺) cell density was decreased relative to NAWM at all timepoints and remained so even at 56 dpl (NAWM ($n = 22$) vs 56dpl ($n = 5$), Tukey's $p = 0.003$) (**Fig 4A,4E**). There were no statistically significant differences between timepoints in Olig2⁺ cell density (7-56dpl) (One-way ANOVA, $F(3, 18) = 0.98$, $p = 0.42$). Importantly, the density of OPCs and immature oligodendrocytes defined as CC1⁺Olig2⁺ (**Fig 4D**) was maintained, showing no statistically significant differences between groups (One-way ANOVA, $F(4, 28) = 0.88$, $p = 0.49$) (**Fig 4F**), a similar observation to that of MS lesion tissue (**Fig 1A**). However, the proportion of OPCs increased significantly at 7dpl (7 dpl ($n = 4$) vs NAWM ($n = 16$), Tukey's $p < 0.0001$), but returned to that of NAWM by 56 dpl ($n = 5$; Tukey's $p = 0.79$) (**Fig 4G**). Sox2 expression is upregulated by Olig2-defined OPCs following demyelination and corresponds with OPC activation, acting to maintain these cells in a proliferative state and priming them for differentiation⁴. In rodents, OPC activation peaked at 5 dpl⁴. Comparatively, Sox2⁺Olig2⁺ activated OPCs in the rabbit peaked at 14 dpl (Tukey's multiple comparisons test, NAWM ($n = 19$) vs 14dpl ($n = 4$), $p = < 0.0001$), but this peak did not correspond with an increase in total OPC density at 14, 21 or 56 dpl (**Fig 4B-4C**). Likewise, the density of Ki67⁺ proliferating Olig2⁺ OPCs peaked at 14 dpl and declined thereafter, returning to NAWM levels by 56 dpl (**Fig 4J-4K**).

In rodent models, the loss of Sox2 in OPCs and exit from cell cycle correlates with a recovery of mature OL cell density⁴. We analyzed oligodendrogenesis using CC1⁺Olig2⁺ at each time point. Unlike the rodent, the density of mature OLs did not return to NAWM levels. Such that at 56 dpl, the density of post-mitotic OLs remained less than half that of oligodendrocytes in NAWM (Tukey's $p = 0.0013$). However, we noted a significant increase in OL density between 7 and 56 dpl (test for linear trend, 7dpl to 56 dpl, $F(1, 13) = 4.8$, $p = 0.047$) (**Fig 4D, 4L**). Intriguingly, while the overall density of mature OLs was dramatically reduced in the rabbit, the percentage of mature OLs returned to that of NAWM by 56 dpl (NAWM ($n = 16$) vs 56dpl ($n = 5$), Tukey's $p = 0.79$) (**Fig 4M**). These results indicate that while rabbit OPCs remain capable of activation/proliferation and subsequent oligodendrocyte differentiation, the number of OPC recruited following demyelination is insufficient to restore the density of OLs to NAWM levels.

Oligodendrocyte progenitors and large volume demyelination

Compared to rodent models, rabbit lesions exhibited inferior OPC recruitment and delayed OL generation.

We directly compared the dynamics of OPC recruitment and differentiation in the rabbit to analogous rodent focal models of gliotoxin-induced demyelination. The rabbit internal capsule (IC) contained higher density of Olig2⁺ cells compared to mouse spinal cord (SC) (**Fig 5A-B**). Following demyelination, recruitment of Olig2⁺ cells was far more efficient in mouse SC than that observed in rabbit IC (**Fig 5C-D**). To quantitatively compare OPC recruitment and differentiation across models, we compared our results to those of previous lysolecithin models of demyelination in the mouse^{4, 25, 26} and ethidium bromide in the rat caudal cerebral peduncle (CCP)^{4, 9, 27, 28}. Following induction of demyelination, both mouse and rat exhibit increased density of Olig2⁺ following recruitment of OPCs at 5 dpl and thereafter (black and brown lines, **Fig 5E**). However, the relative density of Olig2⁺ oligodendroglia in rabbit does not increase following demyelination and is much reduced compared to mouse and rat (gray line, **Fig 5E-5F**). Similarly, while the density of OPCs in NAWM is greater in the rabbit compared to rat and mouse (**Fig 5G**), the spike in relative OPC density that corresponds with successful recruitment after injury is absent in the rabbit (**Fig 5G-5H**). Both mouse and rat display between two and ten-fold increase overall Olig2 and OPC density relative to NAWM. In contrast, the rabbit OPC response is muted and delayed relative to the other species. Intriguingly, rabbit lesions also display slower OPC differentiation (both absolute and relative) compared to mouse and rat (**Fig 5A-5D, 5I-5J**). Thus, abundant OPC recruitment seems to be a characteristic of the remyelination-efficient mouse and rat models, while less-abundant OPC recruitment is a shared characteristic between the rabbit model and observations of MS lesions (**Supplemental Fig 1A vs Fig 5H**).

Regional differences in axonal injury correspond to chronic gliosis and prolonged OPC activation.

Unlike rodent models with relatively small cross-sectional area, we observed clearly distinct sub-regions within the rabbit lesion. A common feature of rabbit IC lesions was a central core region that displayed a reduced density of axons relative to the rest of the lesion (**Fig 3**). This appears to replicate a common feature of MS lesions which are often found to possess lower axonal density in a central core region²³. We defined the remainder of the lesion as lesion periphery (**Fig 6A-C**). The area of the core region remained stable even at 56 dpl while the area of the lesion periphery significantly reduced with time (**Fig 6G-H**), which could be due to resolution of inflammation, tissue edema, and remyelination.

Oligodendrocyte progenitors and large volume demyelination

At 14 dpl, the lesion core possessed high numbers of both Iba1⁺ microglia and Gfap⁺ astrocytes (**Fig 6D-F**). At this point, the astrocytic and microglial responses were qualitatively similar in the lesion core and periphery. In contrast, at 56 dpl, hypertrophic and reactive astrocytes were highly enriched around the lesion core, often forming a distinct border indicative of glial scar formation (**Fig 2C**). The density of microglia/macrophages (Iba1⁺) was greatest at earlier timepoints and was somewhat reduced in the periphery at later timepoints (**Fig 6D-F, 2D**). Quantification of the density of NonOligo cells reflected these observations indicating that more cell nuclei were present in the core at each stage following demyelination and that cell density gradually decreased following 14 dpl (Two-way ANOVA, location factor, $F(1, 38) = 27.47$, $p < 0.0001$) (**Fig 6I**).

We analyzed the relative recruitment and differentiation of OPCs in both lesion core and periphery (**Fig 7**). The overall Olig2⁺ cell density was significantly greater in the lesion periphery than the core regardless of time (Two-way ANOVA, region main effect: $F(1, 41) = 9.30$, $p = 0.004$) (**Fig 7E**). This difference was seemingly not due to absolute OPC numbers as there was no statistically significant difference between CC1⁺ OPC and immature OL density of the core and periphery across time ($F(1, 32) = 3.01$, $p = 0.85$) (**Fig 7F**). Furthermore, the density of Sox2⁺ activated OPCs (**Fig 7H**) or Ki67⁺ proliferating OPCs (**Fig 7J**) did not differ significantly between regions. However, the proportion of OPCs among the Olig2 fraction consistently exceeded that of NAWM and was greatest in the lesion core ($F(1, 25) = 8.484$, $p = 0.008$) (**Fig 7G, I, K**). At 56 dpl, Sox2⁺ OPCs remained highly abundant representing over half of all OL lineage cells in the core, while the proportion of activated OPCs in periphery had reduced to NAWM levels ($F(1, 32) = 33.24$, $p < 0.0001$). Likewise, while OPC proliferation peaked at 14 dpl in the lesion periphery and decline thereafter, in the lesion core the rate of OPC proliferation continued to rise until 21 dpl and remained substantially higher than NAWM even at 56 dpl ($F(1, 31) = 9.754$, $p = 0.004$). In contrast, the density of CC1⁺Olig2⁺ OL was significantly higher in the lesion periphery across time points ($F(3, 28) = 3.379$, $p = 0.032$) (**Fig 7L**). The proportion of OLs dramatically increased from 7 to 21 dpl in both lesion core and periphery but also trended toward an overall increase in the lesion periphery ($p = 0.058$) (**Fig 7M**). Together, these results suggest that the core environment could be driving OPCs to a state of chronic activation and proliferation following demyelination while the lesion periphery supported OL differentiation after an initial period of activation and proliferation.

Oligodendrocyte progenitors and large volume demyelination

Decreased OL and preserved OPC density in the NAWM directly adjacent to the lesion.

As the larger rabbit lesion afforded a closer examination of regional differences in OPC response to demyelination, we examined the density and proportion of OL lineage cells in and around the border of demyelination. The lesion border was defined by reduced solochrome staining compared to NAWM (**Fig 8A-B**). A region extending 100 μm outside the lesion border was defined as perilesion, while the analogous region within the lesion was classified as the lesion edge (**Fig 8C**). While the perilesion region was not hypercellular, with DAPI density not exceeding NAWM at 7 dpl, the lesion edge exhibited clear hypercellularity that was reduced compared to lesion periphery and core (**Fig 8D, 8I**). Furthermore, the perilesion did not exhibit detectable demyelination as we confirmed that both MBP and FluoroMyelin were not reduced in this region (data not shown). The remaining lesion was divided into inner periphery and lesion core as described above. Intriguingly, the different regions exhibited significantly distinct temporal increases in cellular density ($p = <0.0001$), with the greatest density of cells found in the lesion core at between 14 and 21 dpl (**Fig 8D**). Also corresponding with these demarcations, we observed a distinct pattern of OPC and OL lineage fate between each region (**Fig 8E, 8H**). The density of CC1⁺Olig2⁺ OPC and immature OL cells returned to normal density by 7 dpl within the lesion but was substantially lower in the perilesion region than the NAWM (**Fig 8J**). This suggests that a depletion of OPCs occurs around the site of the lesion as cells are recruited. The density of activated Sox2⁺ OPCs peaked at 14 dpl across regions and was greatest in the central lesion regions (core and inner periphery) (**Fig 8F, 8K**). While the lesion core possessed the greatest proportion of Sox2⁺ OPCs exceeding 50% of Olig2⁺ cells, up to 25% of OL lineage cells expressed Sox2 in the perilesion region ($p = 0.005$) (**Fig 8F, Fig 8L**). This suggests that OPCs outside the region of demyelination upregulate Sox2 in response to nearby demyelination. Intriguingly, the perilesion exhibited a substantial loss of Olig2⁺CC1⁺ OL. In contrast, the perilesion region in mouse spinal cord lesions, which was defined in a similar manner based on the lack of hypercellularity, did not demonstrate OL loss²⁹. As expected, OL loss was apparent within the bulk of the rabbit lesion and the relatively small increase in OL density did not differ significantly between regions ($p = 0.012$) (**Fig 8M-8N**).

Oligodendrocyte progenitors and large volume demyelination

DISCUSSION

The inadequacies of current rodent models of demyelination used to study and develop remyelination-inducing therapies could contribute to their lack of successful translation into the clinic. Here, we show that we are able to produce lesions which can more closely approximate the size of lesions and several key pathological features found in MS patients. Among these, rabbit lesions display insufficient oligodendrocyte progenitor cell (OPC) recruitment where OPC density never exceeds that of NAWM. In stark contrast, regardless of the method of demyelination or species used, small rodent models of demyelination universally display OPC densities between two and ten times above that of NAWM shortly following demyelination. Large volume rabbit lesions were more persistent than those produced in small rodents, with areas of demyelination visible for at least two months. This is consistent with previous observations in the rabbit which noted scant remyelination at two weeks post injection of lysolecithin and chronic demyelination persisting for at least six months³⁰⁻³². Additionally, we found region-specific differences in the response of OPCs to demyelination, notably chronic activation of OPCs in the core and relatively higher OL generation in the periphery.

In small rodent models, OPC recruitment is not considered rate-limiting for remyelination following lysolecithin-induced demyelination^{13, 33}. OL differentiation is dependent on the local density of OPCs both *in vitro*³⁴ and following transplantation into hypomyelinated *shiverer* mice³⁵. This suggests that below a certain threshold local density of OPCs, the process of OL differentiation will be limited. Consistent with this hypothesis, remyelinated (RM) lesions and active lesions in MS, which have a higher propensity for remyelination, tend to have higher densities of OPCs than chronic demyelinated lesions³⁶. Unlike rodent models, the large volume rabbit lesion is associated with inefficient and slow OPC recruitment. Together with the huge difference in volume between rabbit and mouse, this suggests that recruitment may become rate-limiting with increasing lesion volume and that improving recruitment could be a vital and previously underappreciated step in remyelination therapeutics.

While we have observed insufficient OPC recruitment in the setting of larger lesion production, we cannot entirely rule out whether failed OPC recruitment is due to volume or species-dependent differences. There are several well described differences in the behavior of human and mouse OPCs both *in vitro* and following transplantation. For example, adult mouse OPCs are more than twice as migratory compared to adult human OPCs³⁷. Developmentally, there are vast differences in the volume and proportion of myelination required in the human compared to rodents, with developmental myelination completing over years in the human but weeks in rodents³⁵. These differences are also reflected at a cell autonomous level, as, following

Oligodendrocyte progenitors and large volume demyelination

transplantation human OPCs take 8-12 weeks to generate myelin-forming OLs³⁸, while equivalent rodent cells typically complete the process of differentiation in 3-4 weeks³⁹. In contrast, the majority of signaling cascades and transcriptional networks appear to be largely conserved between primary human and mouse OPCs⁴⁰⁻⁴³. Regardless of the contribution of OPC species-differences, the failure of OPC recruitment in the rabbit recapitulates that observed in the majority of MS lesions and thereby provides a suitable test bed for therapeutic approaches to improve the migration and proliferation of OPCs.

Several other causes of remyelination failure have been proposed. Among these, age is a critical factor determining the success for a number of regenerative processes⁴⁴. While young adult animals efficiently remyelinate following focal demyelination and demonstrate widespread OPC recruitment, aged adult animals display substantially impaired rate of remyelination²⁷. In aged adult rodent, OPC recruitment and differentiation are delayed and intriguingly OPC recruitment exhibits a gradient with greatest density in the lesion periphery vs central core²⁷. Future studies will need to investigate the role of aging in the rabbit model to address whether aging similarly influences both recruitment and differentiation in large volume lesions. In addition to age, the innate immune response and extent of axonal injury are critical components that determining the success or failure of remyelination.

Axonal degeneration and transection in MS occurs in the context of inflammatory processes associated with (1) primary demyelination, (2) following the loss of trophic support by myelinating OL and, in some cases, (3) independently of overt demyelination⁴⁵. While loss of trophic support by myelinating OLs results in axonal injury⁴⁶, it is likely that axonal damage in active lesions is due to the intense proinflammatory and toxic microenvironment^{17,47}. Interestingly in experimental models, axonal damage peaks at 6 days following induction of demyelination and decreases substantially thereafter, corresponding to the differentiation of OPCs into myelinating OLs⁴⁸. Further supporting the association of axonal injury and myelin regeneration, induction of OL differentiation and remyelination following ablation of the muscarinic receptor in OPCs prevents axonal loss in EAE⁴⁹. In contrast, an increase in pro-inflammatory signaling such as IFN- γ administered at the time of demyelination prevents efficient OPC recruitment and exacerbates axonal injury⁵⁰. Likewise, in the rabbit model we observed axonal loss and injury marked by App1 following demyelination at 7 dpl. Axonal loss was greatest in the lesion core and the distribution of axonal loss in rabbit lesions was similar to that observed in MS²³. Axonal loss in the rabbit was largely associated with the initial gliotoxin insult as the size of core region remained stable out to 56 dpl and we noted a substantial reduction in App1 staining after 7 dpl. Interestingly, the microenvironment of the core region likely influences OPC state. Within the core

Oligodendrocyte progenitors and large volume demyelination

we observed many more OPCs that remained activated and proliferative for at least two months following demyelination. Prolonged activation of OPCs within the core correlated with increased microglial density suggesting that regional differences in microglial signaling alter OPC state.

Recent studies have increasingly highlighted the role that the innate immune response plays in influencing remyelination⁵¹. OPC recruitment and differentiation is dependent on the polarization of microglia/macrophages, whereby a proinflammatory so-called M1 state promotes OPC recruitment while an immunoregulatory M2 state promotes OPC differentiation and remyelination⁵². The transition of microglia/macrophages to M2 polarization can be blocked by preventing necroptosis of M1 microglia and blocking this transition impairs remyelination of demyelinated cerebellar explants⁵³. While type 1 interferon may recruit microglia into regions of demyelination, type 2 interferon (IFN- γ) produced by Th1 cells is associated with and found at high levels during active lesion formation in MS⁵⁴. Interestingly, IFN- γ directly drives OPC quiescence via upregulation of PRRX1 in human OPCs⁵⁵. This can be reversed by modulation of pharmacological modulation of the local heparanome within regions of demyelination rescuing both remyelination and axonal injury⁵⁰. It is interesting to speculate that in large volume lesions, the transition of the microglia/macrophage response from proinflammatory to immunomodulatory phenotype occurs before the lesion achieves a sufficient density of OPC and thereby instead of promoting remyelination acts instead to limit OPC recruitment resulting in an overall failure of myelin regeneration. Whether or not this occurs in the complex inflammatory milieu of MS lesions will need to be determined.

Remyelination in gliotoxin models provides a simplified model to study the cellular mechanisms of myelin regeneration. While several molecular mechanisms have been described that regulate and are rate-limiting for the repair of mouse and rat lesions, similar studies have not yet been performed in large volume lesions. In the present study, we have shown that aberrations in OPC recruitment and differentiation, akin to those observed in MS, occur following the induction of white matter lesions in the rabbit. These lesions were over 20-fold larger than those produced in mice and exhibit axon injury/loss as well as prolong gliosis that correspond with similar active lesions in MS lesions. OPC recruitment is profoundly different in the rabbit model compared to small rodents as the density of OPCs never exceeds that of NAWM. This corresponds well with observations of OPC density in human MS lesions. Unlike other large species (cat, dog, sheep or pig) with larger volumes of available white matter, rabbits are cost-effective and stereotaxic techniques are routinely accessible in most neuroscience laboratories. As such, we propose that the simplicity and accessibility of the rabbit model along with the clear differences in lesion environment and patterns of OPC recruitment and differentiation will provide a vital

Oligodendrocyte progenitors and large volume demyelination

520 complementary approach for preclinical testing of remyelination therapeutics and aid in the
521 successful development of clinical interventions aimed at promoting myelin regeneration.

Oligodendrocyte progenitors and large volume demyelination

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Oligodendrocyte progenitors and large volume demyelination

FIGURE LEGENDS

Figure 1. Large regions of demyelination following injection of lysolecithin into rabbit subcortical white matter. (A) Diagram representing injection sites, and white matter tract size for comparison with rat and mouse, drawn to scale (5 mm) (mouse, overlay of lab section; rat overlay of rat brain atlas⁵⁷). (B) Lysolecithin-induced lesion in the rabbit stained for FloroMyelin (FM, red) and 4',6'-diamidino-2-phenylindole (DAPI, blue). White dotted line indicates lesion border. (C-D) Maximal cross-sectional area (C) and lesion volume (D) of rabbit lesions at 14 dpl (peak lesion volume, $n = 8$), compared to that seen in lysolecithin mouse models^{18,19}. (E) Maximal cross-sectional area of lesions following lysolecithin injection in rabbit ($n = 6-8$) and rat³³ (dpl, days post-lesion). Mean $\pm$ SEM shown. Scale: 500 μ m.

Figure 2. Evolution of rabbit demyelinating lesions with preserved axon integrity and prolonged gliotic response. (A-D) Representative confocal images of normal appearing white matter (NAWM) stained for myelin, axonal, astrocytic and microglial/macrophage markers. Following demyelination at 7 to 56 days post lesion (dpl), lesions were imaged by widefield epifluorescence (outlined with dotted white line) shown in the left panel. Higher power confocal images of the area outlined with the white box are shown in the right panel. (A) At 7 dpl, myelin basic protein (Mbp, gray) staining demonstrated myelin loss and degeneration within the lesion, and Mbp levels remain depressed over time. (B) Axons, stained with neurofilament (NF, green), persist within the lesion bounds even at 56 dpl. Axonal injury (APP, red) is evident at 7dpl but is reduced thereafter following demyelination. (C-D) Robust neuroinflammatory response, astrocytes were stained with glial fibrillary acidic protein (Gfap, green) and amyloid microglial cells were stained with ionized calcium binding adaptor molecule 1 (IBA1, red). (E) Total DAPI cell density within the lesion was quantified ($n = 4-19$ per group, mean $\pm$ SEM). (F-G) The non-oligodendrocyte lineage cells (nonOligos) was defined as DAPI⁺Olig2⁻ cells. Density (F) and proportion of total cells (G) was quantified ($n = 4-19$). The density of cells increases dramatically following lysolecithin injection and peaks at 14 days. This is largely due to an increase in the density of non-oligodendrocyte lineage cells which likely represents microglia and at later time-points astrocytic influx and proliferation (G). Lesion image scale: 500 μ m. Confocal scale: 25 μ m.

Figure 3 (related to Figure 3). Lesions were composed of an axon deficient core, with higher axon preservation in the periphery. Representative 60x oil immersion objective + constrained iterative deconvolution stitched images (0.8 μ m steps over 4 μ m depth) of NAWM (A) or following lysolecithin injection (B-E) stained for neurofilament (NF, green) and/or DAPI (blue).

Oligodendrocyte progenitors and large volume demyelination

White dotted lines indicate lesion borders and green dotted lines represents lesion cores. Retraction bulbs were observed in and around the lesions at all timepoints (small frame images found in **B-E**). Scale bar: 100um.

Figure 4. Insufficient OPC recruitment and oligodendrocyte generation following demyelination in rabbit white matter. (A) Olig2 (red) was used to identify total oligodendrocyte lineage cell density (NAWM, 14dpl, and 56 dpl shown). Insert shows high magnification of labelled cells. Activated Sox2⁺ OPCs (B, cyan), proliferative Ki67⁺ OPCs (C, gray) and mature CC1⁺ oligodendrocyte (D, green) were identified by colocalization with Olig2. Additionally, Sox2 has been noted to be expressed by a subpopulation of astrocytes⁴, which are identified as Sox2⁺Olig2⁻ cells. (E) Oligodendrocyte lineage cell density was significantly depressed relative to NAWM following demyelination ($n = 4-22$, one-way ANOVA). The density and proportion of OPCs defined as either Olig2⁺CC1⁻ (F-G), Sox2⁺Olig2⁺ (H-I) or Ki67⁺Olig2⁺ (J-K) increased following demyelination and peaked between 7-14 dpl. While the proportion of mature oligodendrocytes (Olig2⁺CC1⁺) (M) increased following demyelination, the overall density remained significantly depressed relative to NAWM (L). Mean $\pm$ SEM. Tukey's multiple comparisons post-test vs. NAWM. **, ***, and **** indicate $p < 0.01$, 0.001 , and 0.0001 , respectively. Large frame scale: 50 μ m. Small frame scale; 20 μ m.

Figure 5. Rabbit lesions show delayed recruitment of OPCs, abundant Sox2 activation, and slower maturation of oligodendrocytes compared to rodent models. Representative images of normal appearing white matter (NAWM) of mouse spinal cord (A) and rabbit internal capsule (B). Mature oligodendrocytes and OPCs identified as Olig2⁺CC1⁺ and Olig2⁺CC1⁻, respectively. Comparative images of lysolecithin induced lesions in mouse (C) and rabbit (D) at 14 dpl. White dashed line indicates lesion border. L indicates lesion area when border is visible. (E-J) Comparison of oligodendrocyte lineage cell response following demyelinating lesions between rabbit (gray), mouse (black), and rat (brown). Density and relative change in density compared to NAWM of that species. (E-F) While Olig2⁺ cell density increases relative to NAWM following lesion production in mouse⁴ and rats⁴, it remains depressed in rabbits. (G-H) Relative to NAWM, OPC density in rat²⁷ (Pdgfra mRNA⁺) and mouse²⁵ (NG2⁺) models increases greatly following lesion production. Conversely, OPC density in the rabbit (CC1⁻Olig2⁺) does not significantly change from NAWM. (I-J) Mature OLs determined by Olig2⁺PLP⁺ (mouse²⁶, black), or Olig2⁺CC1⁺ (rat^{9, 28}, rabbit). Rat mature OL density curve was determined from two separate studies, with NAWM (original data: 326.4 ± 27.1 cells/mm²) and 10dpl (original data: 102.2 ± 14.7 cells/mm²) data from

Oligodendrocyte progenitors and large volume demyelination

Syed et al. 2011 normalized to the results of Neumann et al. 2019 (7, 21, 49 dpl). Mean $\pm$ SEM shown. Scale bar: 100 μ m.

Figure 6. Axonal deficient lesion core does not expand with time following demyelination.

Representative images following demyelination at 14 days post lesion (dpl), stained for axons with neurofilament (NF, green), axonal injury with beta amyloid precursor protein (App, Red), and nuclei with DAPI (blue)(**A**) or astrocytes (Gfap, green), and Iba1⁺ microglia/macrophages (red)(**D**). Lesion border was defined by hypercellularity and FluoroMyelin staining (blue dashed line) and core was defined as a region containing sparse NF⁺ intact axons (green dashed line). (**B-C, E-F**) High power confocal images of the area outlined with the white boxes in **A** and **D**. (**G-H**) Core lesion area did not significantly change through 56 dpl, while peripheral lesion area decreased with time ($n = 5-8$ per group, two-way ANOVA). (**I**) Quantification of non-oligodendrocyte lineage cells (DAPI⁺Olig2⁻ cells). Two-way ANOVA across time points comparing core and periphery indicated a significant difference in the NonOligo cells between core and periphery ($p < 0.0001$), and over time ($p < 0.0001$). The gliotic response was significantly greater in the core compared to the periphery. Sidak's multiple comparisons comparing means of periphery and core at each timepoint. *, **, ***, and **** indicate $p < 0.05, 0.01, 0.001$, and 0.0001 , respectively. Mean $\pm$ SEM shown. Lesion scale bar: 250 μ m. Confocal scale bar: 25 μ m.

Figure 7. OPCs in the axon-sparse core remained activated and proliferative. (A-D)

Representative images following demyelination at 14 days post lesion (dpl), stained for axons with neurofilament (NF, green), activated Olig2⁺Sox2⁺ OPCs (**B**, cyan), proliferative Olig2⁺Ki67⁺ OPCs (**C**, gray) and mature Olig2⁺CC1⁺ oligodendrocytes (OL) (**D**, green). (**E**) Quantification of Olig2⁺ cell density in the core and periphery. Quantification of cell density and proportion of total oligodendrocyte lineage cells for OPCs (**F-G**), activated OPCs (**H-I**), proliferative OPCs (**J-K**), and mature OLs (**L-M**). Dotted lines represent cellular density of NAWM. While few intact axons remained, this did not significantly affect the recruitment of OPCs to the lesion core. However, a significantly greater proportion of OPCs were activated and proliferating in the lesion core compared to the periphery. Two-way ANOVA was performed across all time points (TME: time main effect) and comparing core and periphery regions (RME: regional main effect). Sidak's post-test comparing regions at each time point. *, **, ***, and **** indicate $p < 0.05, 0.01, 0.001$, and 0.0001 , respectively (NAWM: $n = 19-22$, lesion: $n = 4-7$). Mean $\pm$ SEM reported. Scale bar: 200 μ m.

Oligodendrocyte progenitors and large volume demyelination

Figure 8. Decreased density of mature OLs and no change in OPC density in NAWM directly adjacent to demyelinating lesion. Representative images of NAWM (A) and lysolecithin induced lesion at 56 dpl (B-C) in the rabbit stained with solochrome cyanine (myelin, black). Blue dashed line indicates lesion border. PeriLesion region (purple) was defined as an area extending ~100 μ m past the lesion border. Lesion edge (blue) was defined as an area ~100 μ m smaller than the lesion border. Inner periphery (red) was defined as the area between the outer periphery and the core. Core defined as a central area of the lesion where few intact axons were observed. Scale bar: 200 μ m. Lesion cross-sectional columns covering the various regions stained for (D) DAPI, (E) Olig2, (F) activated OPCs (Olig2⁺Sox2⁺), (G) proliferative OPCs (Olig2⁺Ki67⁺) (H) and mature OLs (Olig2⁺CC1⁺). Scale bar, 500 μ m. (I) Total DAPI⁺ cell density was quantified in all regions. Quantification of cell density and/or proportion of total oligodendrocyte lineage cells between regions for OPCs (Olig2⁺CC1⁻, J), Activated OPCs (Olig2⁺Sox2⁺, K-L), and mature OLs (Olig2⁺CC1⁺, M-N). Dotted lines represent cellular densities of NAWM. Two-way ANOVA was performed across all time points (TME: time main effect) and comparing regions (RME: regional main effect). Mean $\pm$ SEM reported (NAWM: n = 19-22, lesion: n = 4-7).

Supplemental Figure 1. OPC density in MS lesions rarely exceeds that of NAWM, but is a universal feature of rodent models. (A) In human MS lesions, OPCs were identified as either Olig2⁺Nogo⁻ (green)³⁶, Olig2^{strong} (blue)⁵⁸ or NG2⁺ cells (orange)⁵⁹. Gray bar indicates the range of observed OPC density from control human white matter (mean $\pm$ 1 SD, n = 5 patients)³⁶. OPC density rarely surpasses that of NAWM. RM = remyelinated lesions. In experimental demyelination, relative OPC density in toxin (B) and immune-mediated models (C) was plotted against time following the induction of demyelination (lysolecithin and ethidium bromide), or appearance of demyelinated lesions (Cuprizone, EAE, Theiler's virus). OPCs were identified as NG2⁺ (Cuprizone⁶⁰, red; EAE^{61, 62}, purple; lysolecithin^{25, 63}, blue; Theiler's virus⁶⁴, orange), or Pdgfra mRNA⁺ (Rat EB²⁷, green), n = 3-5. Horizontal dotted line indicates the normalized OPC density of the mouse. Robust OPC recruitment evidenced by increased OPC density relative to normal white matter was a common feature of all rodent models, regardless of method of demyelination. Rat EB = ethidium bromide induced lesions in the rat. EAE = experimental autoimmune encephalomyelitis. Mean $\pm$ SEM shown.

Supplemental Figure 2 (related to Figure 3). Amoeboid microglia in lesion core form large vesicles surrounded by astrocytic processes. (A-B) Confocal images of NAWM stained for Iba1 (red), Gfap (green), and DAPI (blue). Cells at the center of the frame represent an astrocyte

Oligodendrocyte progenitors and large volume demyelination

841 (A, green arrowhead) or microglial cell (B, red arrowhead), respectively. (C) Orthogonal views of
842 an amoeboid microglial cell within the core of a lesion at 56 dpl. Scale bar: 25 µm.

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844 **Supplemental Figure 3 (related to Methods). Significant post-operative weight loss in the**
845 **rabbit, and depiction of injection site. (A)** Dye injection (Evans Blue in PBS) showing injection
846 site (left hemisphere). Ruler, 1 mm between dashes. (F) Averaged weight of nonlesioned (blue)
847 and lesioned (gray) animals.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Supplemental 1

Supplemental 3

Supplemental 2