Meningeal lymphatic drainage regulates oligodendrocyte survival and brain myelination

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Highlights

- Meningeal lymphatic vessel ablation causes loss of mature oligodendrocytes.
- Demyelination due to meningeal lymphatic ablation relies on a competent immune system.
- Meningeal lymphatic dysfunction affects brain remyelination and aggravates axonal loss.
- Multiple sclerosis patients have decreased VEGF-C levels in the CSF.

SUMMARY

Functional defects in brain drainage by the meningeal lymphatic vasculature have been linked to both altered neuroimmune responses and behavioral deficits. Yet, the specific neurophysiological changes associated with poor meningeal lymphatic drainage remain unclear. Here, we induce meningeal lymphatic dysfunction in two model systems: disruption of vascular endothelial growth factor C/D signaling or genetic ablation of lymphatic endothelial cells. Both show significant gene expression alterations in mature oligodendrocytes precede a prominent reduction in myelin basic protein, oligodendrocyte numbers and specific lipid species. These phenomena were accompanied by increased meningeal adaptive immune cell recruitment and altered myeloid cell activation in the brain. Interestingly, loss of oligodendrocytes due to meningeal lymphatic ablation was only observed in mice capable of mounting competent adaptive and innate immune responses. Further, meningeal lymphatic dysfunction in a model of cuprizone-induced demyelination, provoked a state of immunosuppression in the brain that led to delayed remyelination, axonal loss, and increased cell apoptosis. Finally, in cerebrospinal fluid of patients with multiple sclerosis, a canonical demyelinating disease, we show decreased levels of a major meningeal lymphangiogenic factor, vascular endothelial growth factor C, , particularly shortly after clinical relapses. Taken together, we show that meningeal lymphatic drainage regulates oligodendrocyte function and brain remyelination, with implications for human demyelinating disease.

INTRODUCTION

The network of lymphatic vessels present in the mammalian dura mater continuously drains fluids, solutes, and immune cells.[1-4] Like their peripheral tissue counterparts, the lymphatic endothelial cells (LECs) of the dura express the characteristic lineage markers, including prospero homeobox 1 (PROX1), vascular endothelial growth factor receptor 3 (VEGFR3), and lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1). Dural LECs form initial capillary lymphatics that later converge into larger collecting lymphatics in the vicinity of the skull base foramina.[1,2,5,6] However, and by contrast to most peripheral tissue lymphatics, the maintenance and function of meningeal lymphatic vasculature is dependent on signaling by the lymphangiogenic vascular endothelial growth factor C (VEGF-C), via its receptors VEGFR2 and VEGFR3.[2,3,7-9]

Through its capacity to drain central nervous system (CNS) content into the cervical lymph nodes (LNs), the meningeal lymphatic system has been shown to have a major impact on neuroimmune surveillance, in shaping immune responses to neurotropic infectious agents, and by modulating neuroinflammation in varied CNS disease models.[1,3,10-16] Studies have shown that disrupting meningeal lymphatic drainage, by either ablation of the lymphatic vessels in the dura or cervical lymphadenectomy, modulates CNS demyelinating disease outcome by affecting dendritic cell and myelin-specific T cell responses.[10,15,17,18] Aging *per se* leads to defects in meningeal lymphatic vessel morphology and drainage, and recapitulating the aging-like meningeal lymphatic deficits in adult mice resulted in early cognitive impairment.[3,12,13,19]

However, we know very little about the regulation of specific brain cell responses by meningeal lymphatic vessel drainage, and the impact of early meningeal lymphatic dysfunction on core brain physiological processes, such as lipid homeostasis. Yet, a functional link between lymphatic drainage and lipid recycling in peripheral organs has been comprehensively investigated. The lymphatic vessels in the small intestine villi, called lacteals, mediate the active absorption of digested lipids.[20] In other peripheral tissues, such as the heart and skin, lymphatic vessels work closely with macrophages to mediate the reverse cholesterol transport, where cholesterol is mobilized into lipoproteins which are drained via lymphatics back into plasma for excretion.[21,22] As a consequence, mice that constitutively lack functional peripheral lymphatic vasculature tend to develop obesity.[23,24]

Given these data, and noting the high lipid content of brain myelin sheets,[25] we hypothesized that defective meningeal lymphatic drainage would significantly impact brain lipid composition and myelination. Here, we provide experimental evidence showing a surprising immune-dependent disruption in the function of brain mature oligodendrocytes (MOLs) using two different models of meningeal lymphatic vessel ablation. Importantly, the defects in oligodendrocyte function in mice with ablated meningeal lymphatics led to a delay in brain remyelination upon cuprizone-induced demyelination. Finally, and to correlate these findings with humans diagnosed with a canonical demyelinating disease, we measured VEGF-C in the plasma and cerebrospinal fluid (CSF) of multiple sclerosis (MS) patients and found a decrease in the CSF of these patients when compared to healthy controls.

RESULTS

Reduced meningeal lymphatic coverage by impaired VEGF-C/D signaling precedes a marked loss in MOLs

We took advantage of a well-established experimental model of meningeal lymphatic vessel ablation by abrogation of VEGF-C/D signaling to investigate the effects of reduced lymphatic outflow on the regulation of brain lipid homeostasis. As shown in previous studies, meningeal LECs rely on constant signaling via VEGFR3 for their maintenance, and sequestration of VEGF-C results in loss of meningeal lymphatic vasculature and decreased drainage of CSF into the cervical LNs.[7,8] We confirmed that peripheral injection of adeno-associated virus serotype 9 (AAV9) expressing the extracellular domains 1 to 3 of murine VEGFR3 (10^{11} viral particles), which bind and sequester VEGF-C and -D (VEGF-C/D trap), effectively induced a regression of lymphatic vessels in the brain meninges, when compared to mice injected with control vector (expressing domains 4 to 7 of murine VEGFR3; Figures S1A–S1C). This experimental intervention did not affect meningeal blood vessel coverage or lymphatics in the skin and small intestine (Figures S1B, S1D–S1H). A similar degree of meningeal lymphatic vessel ablation was observed at 5 and 7 weeks upon successive injections of a 10-fold lower AAV9 load (10^{10} viral particles), a regimen implemented to further minimize potential off-target effects of VEGF-C/D sequestration (Figures 1A–1C). Surprisingly, we observed that meningeal lymphatic vessel regression in adult mice at 5 weeks (Figure 1C) preceded a significant decrease in the levels of myelin basic protein (MBP) in the corpus callosum at 7 weeks, but not at 5 weeks (Figures 1D and 1E). This was accompanied by a trend towards decreased MBP fluorescent signal in the motor cortex of the VEGF-C/D trap group at 7 weeks (Figures S1I and S1J). This widespread decrease in MBP after ablation of meningeal lymphatics was further confirmed by western blot measurements using protein extracts from whole forebrains (Figures 1F and 1G). Considering that MOLs are the main source of MBP in the brain,[26] we hypothesized that altered oligodendroglial-lineage cell function directly drove MBP decreased expression in mice with impaired meningeal lymphatic vasculature. Interestingly, mice injected with the VEGF-C/D trap AAV9 showed decreased numbers of MOLs (identified as cells positive for the anti-quaking 7 antibody clone CC1, without expression of platelet derived growth factor receptor alpha, PDGFRα)[27,28] in the corpus callosum at both 5 and 7 weeks (Figures 1H and 1I). A concomitant decrease in MOLs was also observed in the motor cortex at 7 weeks (Figures 1J and 1K). We next asked if the parallel decrease in MBP level and number of MOLs was accompanied by alterations in the brain lipid profile of mice with impaired meningeal lymphatics. An unbiased lipidomic analysis of whole forebrain samples revealed that mice from the VEGF-C/D trap group displayed decreased levels of three lipid classes, namely lactosylceramide (LacCer), bis(monoacylglycerol)phosphate (BMP), and lysophosphatidylserine (LPS), when compared to control mice (Figure S1K). Remarkably, 66 of the 593 lipid species analyzed were found to be significantly decreased in the VEGF-C/D trap group (Figure 1L), including various sphingolipid (such as sphingomyelin, ceramide, dihydroceramide and LacCer), glycerophospholipid (such as phosphatidylethanolamine, lysophosphatidylethanolamine, phosphatidylserine, LPS, BMP, phosphatidylglycerol and phosphatidylcholine), cholesterol ester and triacylglycerol species. Interestingly, decreased levels of phosphatidylcholine, ceramide, and sphingomyelin species have been reported in MS and its experimental autoimmune encephalomyelitis mouse model.[29-32]

Impaired meningeal lymphatic function is linked to pronounced transcriptomic alterations in MOLs

Next, we sought to better understand the cell-specific gene signatures triggered by the loss of meningeal lymphatic drainage, which precede the observed loss of MOLs. To determine this, we collected the forebrains of mice from each group and performed single-cell RNA sequencing (scRNA-seq) at 5 weeks (Figure 2A), a time point that preceded the substantial loss of MOLs in both the corpus callosum and the motor cortex (Figure 1H–1K). We identified 21 cell clusters (Figures 2B, S2A and S2B), whose frequencies were similar in the control and VEGF-C/D trap groups (Figure S2C). Within these, oligodendrocyte precursor cells (OPCs) were identified by their high expression of *Pdgfra* and *Cspg4*, while a population of newly-formed oligodendrocytes was distinguished by the overexpression of *Bcan* and *Itpr2* (Figure S2A).[33-35] Another oligodendroglial-lineage cluster was annotated as myelinating oligodendrocytes due to their intermediate expression levels of *Plp1*, *Opalin* and *Sept4*.[34,35] Based on previously published literature, two clusters of MOLs (expressing the highest levels of *Plp1*) were discriminated based on their expression patterns of *Opalin* (MOLs 1), *Pmp22* and *Klk6* (MOLs 2; Figure S2A).[33-36] Interestingly, amongst all the analyzed forebrain cell types, we observed that the two clusters of MOLs, alongside the two clusters of astrocytes (astrocytes 1 and 2), presented the highest number of differentially expressed genes (DEGs) in mice with impaired meningeal lymphatics (Figure 2C). At this 5-week time point, prior to marked cell loss, genes previously implicated in the regulation of MOL function in physiological or neuroinflammatory conditions, namely *Ptma*, *Opalin*, *Actb*, *Apod*, *Cst3*, and *Uba52* were overexpressed in MOLs from mice with impaired meningeal lymphatic drainage (Figure 2D).[37-43] On the other hand, MOLs also showed a down-regulated expression of several genes previously implicated in CNS myelination/demyelination, including *Tmeff1*, *Zdhhc20*, *Fbxl17*, *Dock5*, *Pde4d*, and *Dcc* (Figure 2D).[42,44-51] Functional pathway analysis involving DEGs between the VEGF-C/D trap and control groups revealed alterations in mitochondrial metabolic processes such as oxidative phosphorylation, oxidative stress, and respiratory electron transport in the MOLs 2 cluster (Figure 2). These data are in accordance with previous studies showing similar metabolic changes in oligodendrocytes prior to demyelination.[52,53] Likewise, the transcriptomic changes in the cluster astrocytes 2 pointed to increased oxidative phosphorylation (Figure S2D). However, unlike MOLs, we detected similar coverage of the corpus callosum by astrocytes expressing glial fibrillary acidic protein (GFAP) in the VEGF-C/D trap and control groups at 7 weeks (Figures S2E and S2F). Comparable numbers of innate myeloid cells expressing ionized calcium-binding adaptor molecule 1 (IBA1) in the corpus callosum, and expression levels of CD68 by these cells, were also observed in the two groups (Figures S2G–S2I). The observed changes in oxidative stress gene pathways in MOLs and astrocytes were further corroborated by the decreased levels of the antioxidant enzyme superoxide dismutase 2 (SOD2)[54,55] in the forebrains of mice with impaired meningeal lymphatic vasculature (Figures 2). However, at this point, the link between increased glial oxidative stress upon meningeal lymphatic ablation and the ensuing loss of MOLs was merely conjunctural, and more experiments were needed to shine light on the possible underlying mechanism(s).

The loss of MOLs is not linked to reduced VEGFR2 signaling or altered OPC responses

We considered the hypothesis that loss of MOLs could be an epiphenomenon linked to reduced VEGF-C/D signaling on oligodendroglial-lineage cells. A closer look at the expression patterns of *Kdr* and *Flt4*, which encode the VEGFR2 and VEGFR3 proteins, respectively, confirmed that both genes are highly expressed by the vast majority of blood endothelial cells (BECs) in the brain.[26] This was also true for the expression of *Vegfc* and *Vegfd* genes (Figure S3A), which encode the two growth factors sequestered and eliminated upon expression of the VEGF-C/D trap molecule. However, surprisingly, we found hardly any expression of *Flt4* within the brain immune and glial cell clusters, and that OPCs expressed the highest levels of *Kdr*, *Vegfc*, and *Ccbe1* (Figure S3B); the later encodes for the collagen and calcium binding EGF domains 1 enzyme that cleaves and activates VEGF-C.[56] To confirm that OPCs express high levels of *Kdr* we performed RNAscope using probes against *Pdgfra*, *Kdr* and *Flt4*, and sections of brains collected at 5 or 7 weeks from mice of the control and VEGF-C/D trap groups (Figures S3C–S3H). This confirmed that OPCs express high levels of *Kdr* (expressed by ~80–100% of all *Pdgfra*$^+$ cells), but do not express *Flt4*, in the corpus callosum and motor cortex. Nearby *Pdgfra*$^-$ brain cells, which we deem to be BECs due to their assembly in vessel-shaped structures, express high levels of both *Kdr* and *Flt4* (Figures S3C and S3F). Of note, the expression levels of *Kdr* in OPCs was not affected by reducing VEGF-C and -D signaling (Figures S3C–S3H). We next sought to determine if OPCs depend on VEGF-C/D signaling for their proliferation and/or maintenance by injecting mice with the DNA intercalating agent 5-ethynyl-2-deoxyuridine (EdU; Figure S3I). However, both the number of nerve/glial antigen 2 (NG2)$^+$PDGFRα^+ OPCs and the rate of OPC proliferation (denoted by the frequency of EdU$^+$NG2$^+$PDGFRα^+ cells) in the corpus callosum and motor cortex were similar between experimental groups at 5 and 7 weeks (Figures S3J–S3O).

To further test whether preventing VEGFR2 signaling alone could mimic the decrease in MOLs observed in the VEGF-C/D trap model, we weekly injected adult mice with anti-mouse VEGFR2 antibodies, or isotype matched antibodies (as a control), for a total of 7 weeks (Figure S3P). Injections with anti-mouse VEGFR2 antibodies did not affect meningeal lymphatic vessel length or the numbers of MOLs but resulted in decreased numbers of OPCs in the corpus callosum and motor cortex (Figures S3Q–S3X). These data indicated that blocking the signaling through VEGFR2 was unable to recapitulate the regression of meningeal lymphatic vessels and loss of MOLs observed in the VEGF-C/D trap model (Figure 1) and strengthen the idea that meningeal lymphatic dysfunction due to reduced VEGF-C/D signaling was at the genesis of MOL loss.

Concomitant loss of MOLs and altered brain immunity are observed in a genetic model of meningeal lymphatic dysfunction

To validate that the changes in brain MOL numbers, myelin proteins and lipids observed in the VEGF-C/D trap model were indeed due to meningeal lymphatic dysfunction, and not artifacts of dampened VEGF-C/D signaling, we took advantage of an inducible genetic model of LEC loss (Figure 3A). Tamoxifen administration to *Cdh5*^{CreERT2} heterozygous mice expressing two *floxed Prox1* alleles (*Cdh5*$^{CreERT2/+}$*;Prox1*$^{flox/flox}$) resulted in a significant decrease in the length of LYVE-1^{+} meningeal lymphatic vessels, without affecting meningeal blood vasculature or peripheral lymphatics (in the skin), when compared to their *Cdh5*$^{CreERT2/+}$ counterparts injected with an identical regimen of tamoxifen (Figures 3B, 3C, and S4A–S4D). Similarly to the VEGF-C/D trap model, we observed a decrease in the number of MOLs in the brains of *Cdh5*$^{CreERT2/+}$*;Prox1*$^{flox/flox}$ mice compared to controls, and no differences in OPC numbers between groups in the motor cortex and corpus callosum (Figures 3D–3F, and S4E–S3G). Interestingly, the degree of meningeal lymphatic vessel coverage was positively correlated with the number of MOLs in both brain regions (Figures 3G and S4H), suggesting an intricate association between reduced meningeal lymphatic function and loss of MOLs. Analysis of corpus callosum samples by transmission electron microscopy revealed increased axonal myelin sheet G-ratio (i.e., axons with thinner myelin sheaths) and decreased percentage of myelinated axons in *Cdh5*$^{CreERT2/+}$*;Prox1*$^{flox/flox}$ mice, compared to controls, but no changes in the number of axons between groups (Figures 3H–3K).

In accordance with prior studies showing a substantial impact of lymphatic drainage on CNS immune responses,[10,12,13] we observed more IBA1^{+} cells with increased levels of CD68 in the lipid-rich corpus callosum of *Cdh5*$^{CreERT2/+}$*;Prox1*$^{flox/flox}$ mice, but not in the motor cortex (Figures S4I–S4N). This was accompanied by a significant increase in T cell recruitment into the leptomeninges (Figures 3L and 3M), a trend towards increased B cells in the dura (Figures S4O–S4R), and a significant expansion of B and T cells in the cervical LNs (Figures 3N–3T) upon genetic ablation of meningeal LECs, without detectable lymphocyte infiltration into the corpus callosum or motor cortex in either group. Similarly, ablation of meningeal lymphatic vessels in the VEGF-C/D trap model also resulted in a trend for higher frequency of B cells in the meningeal dura (Figures S4S–S4V). Overall, these results strengthen the notion that dysfunctional meningeal lymphatic drainage is accompanied by altered immune cell responses in the brain, its meningeal borders and draining lymphoid tissues, and led us to further investigate the roles of such immune alterations induced by lymphatic vessel ablation on oxidative stress levels and MOL loss in the brain.

Loss of MOLs upon meningeal lymphatic vessel ablation is abrogated in immunodeficient mice

First, we tested whether the heightened adaptive immune responses observed upon meningeal lymphatic vessel ablation were mediating the reductions in SOD2 and MOLs in the brain. To investigate this, we injected recombination activating gene 2 deficient (*Rag2*$^{-/-}$) mice, which lack CNS-associated mature T and B cells,[57-59] with the control AAV9 or the AAV9 expressing the VEGF-C/D trap (Figure 4A). Surprisingly, despite efficient meningeal lymphatic vessel ablation (Figures 4B and 4C), *Rag2*$^{-/-}$ mice from the VEGF-C/D trap group showed similar levels of SOD2 in the brain (Figures 4D and 4E), MBP coverage in the corpus callosum (Figures 4F and 4G), and numbers of MOLs in the corpus callosum and motor cortex (Figures 4H, 4I, S5A and S5B), when compared to their *Rag2*$^{-/-}$ control counterparts.

Next, we sought to investigate the involvement of innate immune cells, including microglia, as these are known to play a role in myelin maintenance.[60,61] To achieve this, mice were fed a diet containing the colony-stimulating factor 1 receptor inhibitor PLX5622 at 600 parts per million from week 3 onwards (Figure 4J), a dosage that promoted the ablation of roughly half of the IBA1^{+} cells in the brain (Figures S5C and S5D) and avoided possible confounding effects observed upon a more severe deletion of brain-resident innate myeloid cells.[62,63] In accordance with our findings involving *Rag2*$^{-/-}$ mice, depletion of CNS-associated innate immune cells by PLX5622 also prevented the decrease in SOD2, and abrogated the reductions in MBP and MOLs in mice with meningeal lymphatic dysfunction (Figures 4K–4R, S5E and S5F).

Overall, these results suggest that defective meningeal lymphatic drainage is linked to the generation of deleterious brain-associated immune responses that are unfavorable to MOLs and myelin maintenance.

Meningeal lymphatic vessel ablation leads to immunosuppression, delayed remyelination and worsened axonal loss after brain demyelination

Having observed the impact of impaired meningeal lymphatic function on demyelination in adult mice, we asked if remyelination processes were also affected, using the well-established model of cuprizone-induced demyelination.[64] This model was selected due to its reproducibility, the well characterized process of spontaneous remyelination upon withdrawal of the cuprizone from the diet, and lack of encephalitogenic lymphocyte recruitment into the brain, which could otherwise mask the effects of the meningeal lymphatic vessel manipulations.[64-66] After exposure to the cuprizone diet for 4 weeks (Figure S5G), mice from the control and VEGF-C/D trap groups showed similar levels of demyelination, denoted by the marked decrease in the numbers of MOLs in the corpus callosum and motor cortex (Figures S5H–S5K). To study the effects of meningeal lymphatic vessel ablation on brain remyelination, mice from the control or VEGF-C/D trap groups were fed the cuprizone diet for 4 weeks and then transitioned to regular chow for an additional 2 or 4 weeks (Figure 5A). After confirming the efficiency of the meningeal lymphatic ablation at each time-point (Figures S6A and S6B), we quantified the expression of MBP in the corpus callosum and motor cortex. Interestingly, we found significantly lower levels of MBP and reduced numbers of MOLs in the VEGF-C/D trap group at 2 weeks, when compared to its respective control group (Figures 5B–5E, S6C–S6F). However, by 4 weeks, both groups presented similar levels of MBP and numbers of MOLs in the corpus callosum and motor cortex (Figures 5B–5E, and S6C–S6F), suggesting that meningeal lymphatic dysfunction delays, but does not fully prevent, the replenishment of the pool of MOLs after demyelination by cuprizone. The reduced numbers of MOLs in mice of the VEGF-C/D trap group at 2 weeks was accompanied by deficits in several LacCer, phosphatidic acid, phosphatidylserine, and LPS lipid species (Figure S6G). Of note, both experimental groups showed equal numbers of OPCs at 2 and 4 weeks of remyelination (Figures S6H–S6K). Electron microscopy analysis of the corpus callosum at week 2 of remyelination revealed similar myelin sheet G-ratios and percentage of myelinated axons between the VEGF-C/D trap and control groups (Figures 5F–5H). However, alongside the reduced numbers of MOLs, mice with ablated meningeal lymphatic vessels presented significant axonal loss in the corpus callosum (Figure 5I) and higher levels of caspase-3 in brain protein extracts (Figures 5J and 5K), denoting increased cell apoptosis at 2 weeks of remyelination. Yet, these changes were not accompanied by alterations in GFAP$^+$ astrocyte coverage, IBA1$^+$ myeloid cell numbers, or CD68 expression by IBA1+ cells in the corpus callosum (Figures S6L–S6P).

Because cuprizone-induced demyelination occurs independently from peripheral lymphocyte recruitment and infiltration into the brain,[66,67] we again took advantage of this model to test the hypothesis that the delayed remyelination in mice with a defective meningeal lymphatic vasculature would be rescued by preventing the generation of ensuing deleterious immune responses in the brain. To test this, we injected wild type or immunodeficient *Rag2*$^{-/-}$ mice with either the control or VEGF-C/D trap AAV9 and subjected all the mice to the same remyelination paradigm after cuprizone exposure (Figure 5L). Surprisingly, the delay in MOL replenishment at week 2 of remyelination in the corpus callosum of wild type mice treated with the VEGF-C/D trap was not recapitulated in *Rag2*$^{-/-}$ mice (Figures 5M and 5N), and was linked to the generation of an immunosuppressive response characterized by decreased levels of cytokines and chemokines in the brain, including IL-33, IL-1α, IL-22, C-X-C motif ligand 1, and C-C motif ligand 2 (Figure 5O). Of note, the concomitant reduction in MOLs in the corpus callosum and deleterious immunosuppressive response induced by meningeal lymphatic dysfunction were largely absent in *Rag2*$^{-/-}$ mice, but not in mice treated with PLX5622 during the remyelination phase (Figures S6Q–S6T).Taken together, these results suggest that impaired meningeal lymphatic drainage delays the process of brain MOL generation after demyelination, and worsens axonal loss, through a process that seems to be independent of OPCs and is linked to the establishment of a repressed neuroinflammatory environment promoted mainly by adaptive immune cells.

VEGF-C levels in the CSF from MS patients decrease shortly after a relapse

Our results using mouse models suggest that impaired meningeal lymphatic function is associated with abnormal immune responses that lead to defective brain myelination and remyelination. As a way to assess potential alterations in lymphatic-related biomarkers in humans with CNS demyelination,[68] we next decided to measure the levels of VEGF-C in the plasma and CSF from MS patients and age-matched healthy controls (Table S1). The levels of VEGF-C in the plasma were comparable between groups (Figure 6A). However, MS patients showed significantly lower levels of VEGF-C in the CSF when compared to controls (Figure 6B and S6U). VEGF-C levels in the CSF remained significantly different between MS and healthy control patients even after controlling for sex, age and the interaction between sex and diagnosis (Figure S6U). Of note, MS patients that experienced a relapse less than 60 days before sample collection showed even lower CSF VEGF-C levels when compared to patients that were relapse-free for longer than 60 days (Figure 6C).

DISCUSSION

The meningeal lymphatic vasculature mediates CSF drainage and modulates CNS-related immune responses, both at the level of the meningeal tissue and the brain parenchyma.[3,10,12,13] Interestingly, reduced meningeal lymphatic drainage, either in adult or aged mice, has been linked to dampened fluid flow through the glymphatic system, altered gene expression signatures in whole hippocampal tissue and the appearance of mild cognitive deficits.[3,13] However, until now, little was known about the impact of reduced meningeal lymphatic drainage on the function of specific brain cell types, and on steady state neurophysiological processes such as myelination. Herein, we have found that meningeal lymphatic vessel dysfunction, induced either via VEGF-C/D depletion or the targeted genetic ablation of dural LECs, culminated in MOL loss, and, consequently, reduced levels of MBP and numerous lipid species in the brain.

Impairing meningeal lymphatic drainage led to significant transcriptomic alterations in MOLs that preceded overt cell and lipid loss. Down-regulation of the gene *Dcc,* which encodes for a netrin-1 receptor, might point to deficits in MOL process extension, branching and myelin membrane sheet formation.[44] Additionally, the down-regulation of *Fbxl17,* a gene involved in the regulation of oxidative stress,[69] further supports the notion of increased oxidative stress in MOLs from mice with reduced meningeal lymphatic function. Of note, FBXL17 protein levels are reduced in the plasma of patients with pediatric MS.[49] MOLs from mice with defective meningeal lymphatics also showed an overexpression of the *Apod* and *Cst3* genes. Apolipoprotein D (encoded by *Apod*) has been found to be increased around infarct areas in a model of middle cerebral artery occlusion, whereas *Cst3* overexpression by oligodendrocytes containing alpha-synuclein inclusions as been reported in a model of multiple system atrophy.[37,38] Meningeal lymphatic dysfunction by depletion of VEGF-C/D also led to decreased expression of the genes *Tmeff1* and *Pde4d* in MOLs, resembling what was observed upon extensive demyelination in a model of ALS and in the cuprizone model.[50,51,70] Interestingly, and in line with the alterations observed in white matter oligodendrocytes from MS patients, MOLs from mice with impaired meningeal lymphatic function presented a down-regulation of *Zdhhc20* and *Dock5*, and an up-regulation of *Actb* and *Ptma*.[42] Likewise, upon ablation of meningeal lymphatic vessels, MOLs overexpressed *Opalin*, which was found to be equally overexpressed during remyelination in MS and essential for efficient oligodendrocyte myelination during development and in response to injury.[39-41] Notwithstanding the correlative nature of these observations, these scRNA-seq data suggests that, shortly upon meningeal lymphatic vessel ablation, the murine brain MOLs acquire a gene expression signature of enhanced oxidative stress that precedes cell loss and resembles the oligodendrocyte transcriptomes detected in mouse models and humans that present blatant demyelination. This conclusion is further supported by the decreased levels of the antioxidant SOD2[54,55] in the brains of mice with defective meningeal lymphatics.

Astrocytes from the brains of mice with ablated meningeal lymphatic vessels also showed marked transcriptomic alterations that preceded the loss of MOLs. However, unlike MOLs, this was not associated with loss of GFAP$^+$ astrocytes. Nevertheless, considering that astrocytes can modulate oligodendrocyte responses and brain remyelination upon different insults,[71,72] it will be important to explore whether astrocytic (dys)function is involved in the meningeal lymphatic-induced defects in demyelination described herein.

Of note, our data suggests that the deficit in MOLs upon induction of meningeal lymphatic dysfunction by depletion of VEGF-C/D is not due to off-target effects on OPC proliferation and differentiation, or to lack of appropriate VEGFR2 signaling in oligodendroglial-lineage cells, despite the observed decrease in OPCs upon prolonged VEGFR2 blockade. Supporting previously published data,[73,74] we found that OPCs in the adult mouse brain express high levels of *Kdr*, encoding for VEGFR2, but not of *Flt4*, encoding for VEGFR3. Expression of VEGFR3 by neural progenitor cells, namely OPCs, has been detected during developmental stages and proven to be essential for VEGF-C-mediated OPC proliferation.[75] However, it is possible that, as brain development ensues and mice reach adulthood, OPCs down-regulate the expression of *Flt4*, but maintain higher expression levels of *Kdr*. Our data showing decreased numbers of OPCs upon prolonged antibody-mediated blockade of VEGFR2 is in accordance with the reported loss of optic nerve OPCs during embryonic stages in *Vegfc*-deficient mice.[75] Despite the apparent resilience of MOLs to VEGFR2 signaling blockade, based on previously published data[73-75] and on our new results, it will be interesting to explore the effects of altered VEGFR2 signaling on OPC function in the adult brain.

Using a genetic model of meningeal LEC ablation, we show that increased adaptive immune cell recruitment into the brain meninges and expansion at the brain-draining cervical LNs accompany the marked loss of MOLs and demyelination in the brain. Our observations are in accordance with published studies showing increased numbers of T and B cells in meningeal tissues after lymphatic vessel ablation, and amplified dural T cell recruitment and microglial activation in a mouse model of brain amyloidosis with reduced C-C chemokine receptor type 7-dependent lymphatic egress.[8,10,12,13] The observed recruitment of T and B cells into the murine meninges upon meningeal lymphatic vessel ablation is also in line with the reported formation of meningeal ectopic lymphoid tissues in mouse models of spontaneous MS and in MS patients.[76-79] Interestingly, we show that the decreased survival of MOLs and delayed remyelination upon cuprizone in mice with dysfunctional meningeal lymphatics is not recapitulated in PLX5622-treated or immunodeficient *Rag2*$^{-/-}$ mice. Altogether, these data strongly suggest that impaired meningeal lymphatic drainage promotes a deleterious immune cell gain-of-function at the brain and its border tissues that drives MOL loss and poor brain myelination.

In line with this, a recently published study shows significant alterations in the transcriptome of oligodendrocytes, but not of astrocytes and microglia, in *Rag2*$^{-/-}$ mice, which suggests that a direct crosstalk between adaptive immune cells and oligodendrocytes is essential for maintenance and function of these brain parenchymal glial cells.[80] However, it remains unclear whether the effects of reduced meningeal lymphatic drainage on brain myelination can also be attributed to decreased drainage of, and altered tolerance to, CNS-derived antigens, like certain immunogenic lipid species and myelin components. Future studies should be designed to understand how prolonged meningeal lymphatic dysfunction disrupts the mechanisms of tolerance to CNS antigens, favoring the development of deleterious (an potentially encephalitogenic) T and B cell responses at the draining cervical LNs and meninges that could ultimately mediate MOL stress and poor brain (re)myelination.

We demonstrate that the process of MOL regeneration after cuprizone is delayed in the motor cortex and corpus callosum of mice with reduced meningeal lymphatic drainage. This was, once again, independent of changes in the numbers of OPCs in the two regions. Surprisingly, we show that meningeal lymphatic vessel dysfunction is also linked to worsened axonal loss at the corpus callosum after cuprizone, increased levels of the proapoptotic caspase-3 protein and, more importantly, to lower levels of cytokines and chemokines in the forebrain of immunocompetent mice during remyelination. Importantly, this immunosuppressive environment that is unfavorable for brain remyelination is abrogated in mice that also have a defective meningeal lymphatic vasculature but are unable to mount T and B cell effector responses. This shows once more that the meningeal lymphatic system is essential for the preservation of a neuroimmune environment in adult mice that is essential for appropriate MOL function and an efficient process of brain remyelination. Accordingly, experiments involving *Rag1*$^{-/-}$ mice have shown that T and B cells play a central role in spontaneous remyelination of CNS axons after lysolecithin-induced demyelination in the spinal cord.[81] Moreover, it was recently shown that changes in cytokine levels if the brain, namely tumor necrosis factor and interferon-gamma, dampened the remyelination capacity of oligodendrocytes.[82] Based on these evidence and our new data, it is reasonable to consider that a disruption of meningeal lymphatic drainage leads to altered T and B cell cytokine and chemokine secretion at the meninges, which in turn will affect brain innate immune function, and lead to the formation of a repressed immune environment that constrains the process of remyelination and leads to increased cell death. In agreement, recent work has described a pathogenic interaction between peripherally recruited CD8^{+} T cells, microglia and oligodendrocytes that seems to be at the genesis of brain white matter abnormalities in aging.[83,84] In fact, it is well established that the aged CNS shows impaired myelination, or capacity to remyelinate after an insult.[83,85,86] Of note, aged mice also present decreased meningeal lymphatic vessel coverage, reduced CSF drainage into deep cervical LNs, and a concomitant increase in the numbers of effector T cells in the meninges.[3,12,87] The fact that none of the aging-related oligodendrocyte gene signatures were present in the stressed MOLs that we have isolated from the brains of adult mice with ablated meningeal lymphatics, means that different immune mechanisms might be at play in adult and old mice. It will be interesting to determine if enhancing meningeal lymphatic function in geriatric mice is capable of rejuvenating brain immunity and enhance the aged brain's (re)myelination capacity. Future experiments should also focus on characterizing the precise nature and timeline of the immune interactions and mechanisms that are initiated by meningeal lymphatic vessel ablation in adult mice and culminate in the poor generation of new MOLs and exacerbated axonal loss. This is especially relevant considering that we observe similar numbers of MOLs in mice with intact or ablated meningeal lymphatics at 4 weeks of remyelination. It will be important to test whether there is a rebalance of meningeal adaptive immunity, and of the levels of inflammatory mediators in the brain, at the later stages of the spontaneous remyelination process that is no longer dependent on the status of the meningeal lymphatic system. We have observed decreased levels of VEGF-C in the CSF of treatment-naïve MS patients, particularly within 60 days of a relapse episode. Interestingly, the decreased levels of VEGF-C seem to be limited to the CNS of MS patients, and follow the same trend of VEGFR3, which is also reduced in the MS brain.[88] Considering our data collected from the experiments involving mice, where we show that meningeal, but not peripheral, lymphatics regress after sequestration of VEGF-C/D, one could argue that a similar meningeal lymphatic vessel loss might be observed in MS patients due to reduced expression of both VEGFR3 and its ligand VEGF-C. In fact, in a recently published study, authors use state-of-the-art magnetic resonance imaging modalities to show that the meningeal lymphatic flow is decreased in patients diagnosed with neuromyelitis optica spectrum disorder with acute attack, a CNS demyelinating disease also characterized by exacerbated neuroinflammation.[89] The fact that decreased VEGF-C levels in the CSF are observed soon after a relapse, indicates that exacerbated neuroinflammation does not favor meningeal lymphatic function. The negative effects of inflammation on meningeal lymphatics have also become evident in mouse models of aging, traumatic brain injury and CNS infection by different strains of neurotropic viruses, where drastic meningeal lymphatic vessel morphological alterations culminate in reduced drainage of CSF into the deep cervical

LNs.[11,14] In future clinical studies, it will be vital to include state-of-the-art magnetic resonance imaging approaches[4,5,90] to assess if patients with decreased VEGF-C also present defective meningeal lymphatic vessel morphology and flow of CSF into the cervical LNs, and whether these phenomena are correlated with the level of neuroinflammation and/or the degree of demyelination in the brain, particularly in regions adjacent to the CSF-filled ventricles.[91-93] It will also be important to evaluate the diagnostic and/or prognostic value of measuring VEGF-C in the CSF, either in MS or in other CNS demyelinating disorders.

Limitations of the study

We are describing a novel and clear functional link between the meningeal lymphatic vasculature and oligodendrocyte homeostasis in the adult CNS. Our data also shows that a deleterious gain-of-function by adaptive and innate immune cells is mediating the loss of MOLs and delayed MOL replenishment after a demyelinating insult in mice with dysfunctional meningeal lymphatics. Future experiments should be designed to characterize in greater depth the T and B cell responses at the brain, meninges and cervical LNs in $Cdh5^{CreERT2/+};Prox1^{flox/flox}$ mice, and to determine which specific molecular cues can be targeted, either on immune cells, oligodendroglial-lineage cells, or both, to prevent the defective brain myelination due to meningeal lymphatic dysfunction. We do not provide evidence for the cause, or consequence(s), of reduced VEGF-C levels in the CSF of MS patients. Follow-up studies ought to further assess why MS patients present lower VEGF-C in the CNS and attempt to establish correlations between the levels of VEGF-C in the CSF and other clinical and/or pathological measurements. Finally, it will be important to validate our findings using samples from independent cohorts composed of healthy control and MS patients.

STAR METHODS

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Supplemental information can be found online at https://doi.org/

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AUTHOR CONTRIBUTIONS

S.P.D.N. designed and performed the experiments, analyzed and interpreted most of the data, created the figures and wrote the manuscript; N.D. optimized the RNAscope technique and performed experiments; Y.R. stored, processed and analyzed the scRNA-seq data; C.S.C. and M.M. performed and analyzed the ELISA for human VEGF-C; F.A. assisted with lipidomic data analysis; M.J.B., G.S., S.R., R.S., and A.F. participated in different experimental procedures and analyzed data; J.L.T. and P.J.M. provided resources and intellectual contribution; T.G.O., S.I., and F.P. provided resources, supervised data analysis and provided intellectual contribution; S.D.M. provided most of the resources, designed and performed experiments, provided intellectual contribution, oversaw data analysis and interpretation, and wrote the manuscript.

DECLARATION OF INTERESTS

S.D.M. is listed as an inventor in patent applications concerning modulating lymphatic vessels in neurological disease (University of Virginia Licensing & Ventures Group, and PureTech Ventures LLC). S.R.I. has received honoraria/research support from UCB, Immunovant, MedImmun, Roche, Janssen, Cerebral therapeutics, ADC therapeutics, Brain, CSL Behring, and ONO Pharma, and S.R.I. receives licensed royalties on patent application WO/2010/046716 entitled “Neurological Autoimmune Disorders” and has filed two other patents entitled “Diagnostic method and therapy” (WO2019211633 and US-2021-0071249-A1; PCT application WO202189788A1) and “Biomarkers” (PCT/GB2022/050614 and WO202189788A1). The authors have no additional financial interests to declare.

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STAR METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
LYVE-1 AF488, clone ALY7	Thermo fisher scientific	Cat# 53-0443-82
LYVE-1 eF660, clone ALY7	Thermo fisher scientific	Cat# 50-0443-82
CD31/PECAM1, clone 2H8	Millipore	Cat# MAB1398Z
Quaking 7 (Ab-7), clone CC1	Millipore	Cat# OP80
PDGFRα	R&D Systems	Cat# AF1062
NG2	Millipore	Cat# AB5320
MBP, clone DENPVV	Abcam	Cat# ab7349
CD68, clone FA-11	Biolegend	Cat# 137002
IBA1	Abcam	Cat# ab5076
GFAP	Agilent, Dako	Cat# Z033429-2
CD3 eF660, clone 17A2	Thermo Fisher Scientific	Cat# 50-0032-82
Donkey anti-goat AF488	Thermo Fisher Scientific	Cat# A-11055
Donkey anti-rabbit AF488	Thermo Fisher Scientific	Cat# A-21206
Goat anti-Armenian hamster AF594	Jackson Immunoresearch	Cat# 127-585-160
Donkey anti-goat AF594	Thermo Fisher Scientific	Cat# A-11058
Donkey anti-mouse AF594	Thermo Fisher Scientific	Cat# A-21203
Donkey anti-rabbit AF594	Thermo Fisher Scientific	Cat# A-21207
Donkey anti-rat AF647	Thermo Fisher Scientific	Cat# A-48272
Donkey anti-goat AF647	Thermo Fisher Scientific	Cat# A-21447
β-actin, clone 8H10D10	Cell Signaling	Cat# 3700
Cleaved caspase-3	Cell Signaling	Cat# 9661
SOD2, clone E-10	Santa Cruz Biotechnology	Cat# sc-137254
α-tubulin		
Mouse anti-rat IgG2a H&L (horseradish peroxidase), clone KT97	Abcam	Cat# ab106783
Horse anti-mouse IgG (horseradish peroxidase)	Cell Signaling	Cat# 7076
Goat anti-rabbit IgG (horseradish peroxidase)	Cell Signaling	Cat# 7074
Zombie Aqua	Biolegend	Cat# 423102
CD45-PerCP-Cy5.5, clone 30-F11	BD Biosciences	Cat# 550994
CD19-FITC, clone 6D5	Biolegend	Cat# 115506
CD19-APC-Cy7, clone 6D5	Biolegend	Cat# 115530
TCRβ-BV711, clone H57-597	BD Biosciences	Cat# 563135
CD4-PE-Dazzle 594, clone GK1.5	Biolegend	Cat# 100456
CD4-PE-Cy7, clone GK1.5	Biolegend	Cat# 100422
CD8α-PB, clone 53-6.7	BD Biosciences	Cat# 558106

CD16/32, clone 93	Biolegend	Cat# 101302
Mouse Strains		
C57BL/6J	The Jackson Laboratory	Stock# 000664
B6.Cg-Rag2$^{tm1.1Cgn}$/J	The Jackson Laboratory	Stock# 008449
C57BL/6-Tg(Cdh5-cre/ERT2)1Rha	Taconic Biosciences	Stock# 13073
Prox1^{tm2Gco}	Kindly provided by Dr. Guillermo Oliver	N/A
Mouse Treatments and Diets		
Rat IgG1, clone HRPN (isotype control)	Bio X Cell	Cat# BE0088
Rat IgG1 anti-mouse VEGFR2, clone DC101	Bio X Cell	Cat# BE0060
Tamoxifen	Sigma-Aldrich	Cat# T5648
0.2% cuprizone supplemented diet, with red dye	Envigo	Cat# TD.140804
PLX5622 supplemented diet (600 ppm), with 15 kcal % fat, with blue dye	Research diets	Cat# D21102810
Control diet, with 15 kcal % fat, no dye	Research diets	Cat# D11112201N
EdU (5-ethynyl-2-deoxyuridine)	Thermo Fisher Scientific	Cat# E10187
Plasmids		
psubCMV-mVEGFR3-d1–3-Fc-WPRE	Kindly provided by Dr. Jean-Leon Thomas	N/A
psubCMV-mVEGFR3-d4–7-Fc-WPRE	Kindly provided by Dr. Jean-Leon Thomas	N/A
Human Biological Samples		
Human CSF and plasma	Karolinska University Hospital	Table S1
Chemicals		
Corn oil	Sigma-Aldrich	Cat# C8267
*InVivo*Pure pH 7.0 Dilution Buffer	Bio X Cell	Cat# IP0070
PLX5622	Chemgood	Cat# C-1521
DirectPCR (tail)	Viagen	Cat# 102-T
Ultra pure water	Apex Bioresearch Products	Cat# 18-195
Agarose LE	Apex Bioresearch Products	Cat# 20-102
Apex Safe DNA Gel Stain	Apex Bioresearch Products	Cat# 20-275
RNAscope Target Probe *Flt4*	Advanced Cell Diagnostics	Cat# 481371
RNAscope Target Probe *Kdr*	Advanced Cell Diagnostics	Cat# 414811-C2
RNAscope Target Probe *Pdgfra*	Advanced Cell Diagnostics	Cat# 480661-C3
Opal 520 reagent	Akoya	Cat# OP-001001
Opal 620 reagent	Akoya	Cat# OP-001004
Opal 690 reagent	Akoya	Cat# OP-001006

RNAscope® Multiplex TSA buffer	Advanced Cell Diagnostics	Cat# 322809
10% neutral buffered formalin	Epredia	Cat# 5701
10x phosphate buffered saline (PBS) pH 7.4	Fisher BioReagents	Cat# BP-399
Heparin	Fisher BioReagents	Cat# BP2425
Triton X-100	Sigma-Aldrich	Cat# X100
Sucrose	Cargill	Cat# 62-112
Gelatin	Sigma-Aldrich	Cat# G1890
Chromium (III) potassium sulfate dodecahydrate	Sigma-Aldrich	Cat# 243361
10x citrate buffer	Sigma-Aldrich	Cat# C9999
Tissue-Plus O.C.T. Compound Clear	Fisher HealthCare	Cat# 4585
4,6-diamidino-2-phenylindole (DAPI)	Thermo Fisher Scientific	Cat# 62248
Immu-Mount	Epredia	Cat# 9990402
Bovine serum albumin	Genesee Scientific	Cat# 25-529
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	Cat# 276855
Tween 20	Bio-Rad Laboratories	Cat# 1706531
10x Tris/Glycine/SDS buffer	Bio-Rad Laboratories	Cat# 1610772
10x tris buffered saline (TBS)	Bio-Rad Laboratories	Cat# 1706435
T-PER Tissue Protein Extraction Reagent	Thermo Fisher Scientific	Cat# 78510
UltraPure 0.5 M ethylenediaminetetraacetic acid	Thermo Fisher Scientific	Cat# 15575020
Halt protease inhibitor cocktail	Thermo Fisher Scientific	Cat# 78430
Phosphatase inhibitor cocktail	Cell Signaling	Cat# 5870
Phenylmethylsulfonyl fluoride	Cell Signaling	Cat# 8553
Pierce Coomassie Plus Assay Kit	Thermo Fisher Scientific	Cat# 23236
Laemmli Sample Buffer	Bio-Rad Laboratories	Cat# 1610737
Precision Plus Protein Kaleidoscope Prestained Protein Standards	Bio-Rad Laboratories	Cat# 1610375
β-Mercaptoethanol	Sigma-Aldrich	Cat# M6250
Mini-PROTEAN TGX gels	Bio-Rad Laboratories	Cat# 4561094
Pierce ECL Western Blotting Substrate	Thermo Fisher Scientific	Cat# 32209
Trans-Blot Turbo 5x Transfer buffer	Bio-Rad Laboratories	Cat# 10026938
4% paraformaldehyde in PBS	Boster Bio	Cat# AR1068
4 % paraformaldehyde, 1%	Electron Microscopy	Cat# 15949-70

glutaraldehyde in 0.1 M phosphate buffer, pH 7.2	Sciences	
RPMI 1640	Genesee Scientific	Cat# 25-506
FBS	Corning	Cat# 35-010-CV
Commercial Assays		
Click-iT EdU AF488 Imaging Kit	Invitrogen	Cat# C10337
Adult Brain Dissociation Kit	Miltenyi Biotec	Cat# 130-107-677
Chromium Next GEM Single Cell 3’ GEM Kit v3.1	10x Genomics	Cat# PN-1000123
Library Construction Kit	10x Genomics	Cat# PN-1000190
Chromium Next GEM Single Cell 3’ Gel Bead Kit v3.1	10x Genomics	Cat# PN-1000122
Chromium Next GEM Chip G Single Cell Kit	10x Genomics	Cat# PN-1000127
Dual Index Kit TT Set A	10x Genomics	Cat# PN-1000215
RNAscope® Multiplex Fluorescent Reagent Kit v2	Advanced Cell Diagnostics	Cat# 323100
Human VEGF-C Quantikine ELISA Kit	R&D Systems	Cat# DVEC00
ZymoPURE Plasmid Miniprep Kit	Zymo Research	Cat# D4209
ZymoPURE II Plasmid Maxiprep Kit	Zymo Research	Cat# D4203
Deposited Data		
scRNAseq	This paper	GSE230709
Software and Algorithms		
MAST	Finak *et al.*[94]	Version 1.26.0
Metascape	Zhou *et al.*[95]	Version 3.5.20230501
FIJI	Schindelin *et al.*[96], Arshadi *et al.* [97]	Version 2.3.0/1.53q
MyelTracer	Kaiser *et al.*[98]	Version 1.3
FlowJo	BD Biosciences	Version 10.10.0
Prism	GraphPad software	Version 9.5.1

RESOURCE AVAILABILITY

Lead contact

All information and requests for further resources and reagents should be directed to and will be fulfilled by the lead contact: Sandro Da Mesquita (damesquita@mayo.edu).

Materials availability

All requests for resources and reagents should be directed to and will be fulfilled by the lead contact. This study did not generate new unique reagents.

Data and code availability

- The single-cell RNA-seq data reported in Figures 2 and S2 have been deposited at GEO and are publicly available as of the date of publication. The accession number is listed in the key resources table.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND PATIENT SAMPLES

Mice strains and housing

C57BL/6J mice (stock# 000664, The Jackson Laboratory), B6.Cg-Rag2$^{tm1.1Cgn}$/J mice (stock# 008449, The Jackson Laboratory), C57BL/6-Tg(Cdh5-cre/ERT2)1Rhamice (stock# 13073, Taconic Biosciences), and Prox1^{tm2Gco} mice (kindly provided by Dr. Guillermo Oliver, Feinberg School of Medicine, Northwestern Medicine, Northwestern University; these mice were provided in a mixed C57BL/6 and NMRI background)[99] were bred and maintained under standard housing conditions, in ventilated cages, on a 12-hour light/dark cycle (lights on at 6 a.m.), in a temperature- and humidity-controlled environment. Mice were fed with regular rodent chow, unless stated otherwise, and water *ad libitum*. Young adult male mice were used for all experiments, except for experiments using *Cdh5*$^{CreERT2/+}$ and *Cdh5*$^{CreERT2/+}$*;Prox1*$^{flox/flox}$, which included both males and females. The exact ages, sexes and total numbers of mice used in each group are stated in the figure legends. All animal procedures were approved by the Mayo Clinic Institutional Animal Care and Use Committee and were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Patient samples and demographics

Blood samples were collected in ethylenediaminetetraacetic acid (EDTA)-coated tubes, centrifuged at 1,500 g for 15 minutes (min) at room temperature (RT), the resulting plasma was aliquoted and stored at –80°C until further analysis. CSF samples were collected, immediately centrifuged at 350 g for 10 min at RT, and the supernatant was aliquoted and stored at –80°C until further analysis. Original ethical approval was given by Regionala Etikprövningsnämnden I Stockholm 2010-02-16; last amendment by

Etikprövningsmyndigheten (Stockholm avdelning 2 medicin) Dnr 2022-03650-02 (in 2022-07-20). Supplemental Table S1 contains detailed patient demographic information and the corresponding levels of VEGF-C measured in the plasma and CSF.

METHOD DETAILS

AAV9 generation and transduction

The two plasmids, *psubCMV-mVegfr3-d1–3-Fc-WPRE* and *psubCMV-mVegfr3-d4–7-Fc-WPRE* were kindly provided by Dr. Jean-Leon Thomas (co-author of this study) and used for the synthesis of the AAV9-$mVegfr3_{1–3}$-*Ig* (encoding the extracellular domains 1–3 of VEGFR3 fused to a immunoglobulin Fc domain; VEGF-C/D trap group) and the AA9-$mVegfr3_{4–7}$-*Ig* (encoding the extracellular domains 4–7 of VEGFR3 fused to a immunoglobulin Fc domain; control group), respectively. The different AAV9s were generated by Vigene Biosciences (now Charles River Laboratories). For the transduction of each AAV9, young adult mice received either a single intra-peritoneal (i.p.) injection of 10^{11} viral particles, or 2 to 3 i.p. injections of 10^{10} viral particles (in 200 μL of sterile 1x PBS). Schemes with the injection regimens are included in the figures.

Injections with anti-mouse VEGFR2 blocking antibodies

C57BL/6J mice were injected weekly with 100 μg of rat IgG1 anti-mouse VEGFR2 antibodies (clone DC101, BioXCell) or rat IgG1 isotype control antibodies (clone HRPN, BioXCell), in 200 μL of *InVivo*Pure pH 7.0 Dilution Buffer (BioXCell), for a total of 7 weeks.

Tamoxifen injections

Tamoxifen was diluted in corn oil (50 mg/kg, Sigma-Aldrich). All $Cdh5^{CreERT2/+}$ and $Cdh5^{CreERT2/+}$;$Prox1^{flox/flox}$ mice received daily i.p. injections of tamoxifen during 3 consecutive days. The same regimen of consecutive tamoxifen i.p. injections was repeated a week later.

EdU injections

To evaluate cell proliferation, mice were injected (i.p.) daily with 200 μL of EdU (50 mg/kg; Thermo Fisher Scientific) diluted in sterile 1x PBS, for a total of 3 days prior to euthanasia and tissue collection.

Cuprizone and PLX5622 diets

To induce brain demyelination, mice were fed with a diet supplemented with 0.2% cuprizone (Envigo) for 4 weeks. Remyelination was triggered by removing cuprizone from the diet, and transition mice to regular chow diet for an additional 2 or 4 weeks.

To inhibit CSF1R signaling, mice were fed with a diet supplemented with PLX5622 (Chemgood) for a total of 2 or 4 weeks. PLX5622 was incorporated in the diet at 600 ppm (Research diets). Control mice received a nutritionally similar diet, but without PLX5622 (PicoLab Rodent Diet 20) during the same time period.

Tissue collection and processing

Mice received an i.p. injection of pentobarbital (150 mg/kg). Under deep anesthesia, a ~2 cm incision was made along the midline to open the abdominal cavity. The chest was opened by cutting the diaphragm and the ribs upwards along the lateral surface (~2.5 cm cuts on each side). The chest flap was reflected to expose the heart. A small incision (2–4 mm) was made in the right atrium, immediately followed by a slow injection of 20 mL of ice-cold 1x PBS (pH 7.4) with heparin (10 U/mL; Sigma-Aldrich) into the left ventricle, using a 20-gauge needle. For transmission electron microscopy, mice were first perfused with 10 mL of ice-cold 1x PBS (pH 7.4) immediately followed by perfusion with 25 mL of ice-cold 4% paraformaldehyde in PBS (Boster Bio, Pleasanton, CA). Tissues were collected immediately after euthanasia and processed appropriately.

Immunofluorescence stainings and microscopy

The whole ear, a section of small intestine, the dorsal skull cap and either the entire brain or only one brain hemisphere were immersed in 10% formalin (Epredia) and kept at 4ºC with agitation. Skull caps and the whole ears were transferred to 1x PBS after ~12 or ~48 hours, respectively. The small intestine and brain tissues were transferred to 30% sucrose solution in 1x PBS after ~48 hours. Intestine and brain samples were frozen in OCT compound (Fisher HealthCare) 24–36 hours later. Brain sections were cut at a thickness of 50 μm in the cryostat (Leica CM 3050 S), collected into cold 1x PBS in a 24-well plate, and kept at 4ºC until further use. Small intestine sections were cut at a thickness of 30 μm in the cryostat and collected onto gelatin A-coated SuperFrost slides (Fisherbrand). The epidermis and dermis layers of the ear skin were separated immediately before starting the staining. The meningeal tissue (mostly composed of dura and arachnoid) was peeled from the skull cap within one week after fixation and kept in 1x PBS at 4ºC until further use.

Meningeal whole mounts, ear skin tissue, brain, and small intestine sections were permeabilized in 1x PBS 0.5% Triton X-100 (PBS-T; Sigma-Aldrich) at RT for 30 min with agitation. Antigen retrieval was performed only before the double staining with anti-mouse quaking 7 (Ab-7, clone CC1, Millipore) and PDGFRα (R&D Systems). This step consisted in incubating tissue with citrate buffer (Sigma-Aldrich) for 30 min in a water bath at 80ºC; each free-floating section was transferred to a 1.5 mL tube with 500 μL of citrate buffer. Incubation with the primary antibodies against mouse LYVE-1-AF488 (1:150), LYVE-1-eF660 (1:200), CD31/PECAM1 (1:200), CC1 (1:200), PDGFRα (1:200), NG2 (1:200), MBP (1:500), GFAP (1:300), IBA1 (1:200), and CD68 (1:100) was performed overnight at 4ºC with agitation covered from light (for brain sections, meningeal whole mounts, and ear skin) or for 2 hours at RT without agitation (for small intestine sections on slides). Primary antibodies were diluted in PBS-T with 0.5% bovine serum albumin (BSA, Genesee Scientific). After 3 consecutive washes with PBS-T at RT (10 min each), tissues were incubated for 60 min at RT with the appropriate secondary antibodies, all diluted 1:500 in PBS-T, with or without agitation, followed by a single wash with PBS-T at RT (10 min). For CD3-eF660 (1:100) staining in free-floating brain sections, no permeabilization step was performed, the primary antibody was diluted in PBS with 0.5% BSA and incubated overnight at 4ºC with agitation covered from light, and all washes were performed using PBS. Finally, tissues were incubated with 4,6-diamidino-2-phenylindole (DAPI, 1:1000; Thermo Fisher Scientific) for 10 min at RT. After a final wash with 1x PBS for 10 min, slices were mounted onto SuperFrost slides (Fisherbrand, Fisher Scientific), and covered with aqueous Immu-mount (Epredia) and coverslips.

All fluorescence images were acquired using a LSM 880 confocal microscope (Zeiss), except for the images of MBP fluorescence, which were acquired using a BZ-X810 microscope (Keyence). Tiled images obtained using the Zeiss and Keyence microscopes were stitched using each corresponding software. Two images per brain region were used to quantify cell numbers or to measure protein signal coverage in each mouse. Two images of small intestine sections containing LYVE-1$^+$ vessels were taken per mouse. Two images of leptomeningeal tissue were acquired to quantify the numbers of CD3$^+$ cells. The average of the values obtained for each image was calculated and used in further statistical analyses. The remaining quantifications, including the assessments of LYVE-1$^+$ vessels in the skin, MBP coverage and of cells positive for specific mRNA transcripts (using RNAscope), were performed using only one image taken per mouse. The FIJI image processing software was used to determine the cell numbers (Cell Counter plug-in) in the established region of interest (ROI), except for

$CC1^+PDGFR\alpha^-$ cells, that were quantified using Fiji's Analyze Particles plug-in. For this analysis, a threshold was selected for the CC1, PDGFRα and DAPI channels, the PDGFRα signal was subtracted from the CC1 signal using the Image Calculator, and the same plug-in was used to calculate the $CC1^+DAPI^+$ signal. Watershed separation was applied to the $CC1^+DAPI^+$ signal image and a step of automatic particle counting was performed with the Analyze Particles plug-in (size, 9-infinity μm^2; circularity, 0-1). Lymphatic vessel length within a fixed ROI was measured with the FIJI plug-in Simple Neurite Tracer. The coverage percentages of MBP, CD31, GFAP and LYVE-1 were calculated after applying a threshold to the images and using the Measure function of FIJI.

Transmission electron microscopy

The mice brains were collected and one of the hemi-forebrains was sliced coronally, with approximately 1mm thickness, at the corpus callosum/lateral ventricles region. The entire slice was placed in 4% paraformaldehyde, 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2 (Electron Microscopy Sciences, Hatfield, PA). Within one week of tissue collection, samples were sent to the Microscopy and Cell Analysis Core Facility, at Mayo Clinic Rochester, for further processing and imaging. Briefly, after fixation, tissue was washed with PBS, stained with 1% osmium tetroxide, washed in water, stained with 2% uranyl acetate, washed again in water, dehydrated through a graded series of ethanol and acetone, and finally embedded in Spurr resin (Electron Microscopy Sciences). Following a 24-hour polymerization at 60ºC, 0.1 µm ultrathin sections were prepared on T200 copper grids (Electron Microscopy Sciences) and post-stained with lead citrate.

Micrographs were acquired using a JEOL 1400 transmission electron microscope (JEOL, Inc., Peabody, MA) at 80 kV equipped with an AMT Nanosprint12 camera (AMT Imaging, Woburn, MA). A total of 5 images were taken per mouse. The analysis was performed using the MyelTracer software.[98] The number of myelinated and unmyelinated axons was counted on the 5 images, and the average of the values obtained for each image was calculated and used in further statistical analyses. Two of the images were used for the myelin sheet G-ratio analysis, and all the myelinated axons contained within the limits of these two images were analyzed (104-317 axons per mouse). The statistical analysis was performed using the average of all myelin sheet G-ratios per mouse.

Western blot

For western blot analysis, one hemi-forebrain per mouse was dissected, weighted, snap-frozen in dry-ice and kept at –80ºC until protein extraction. The frozen tissue was resuspended in protein extraction buffer (T-Per buffer) containing T-PER Tissue Protein Extraction Reagent (Thermo Fisher Scientific), UltraPure 0.5 M ethylenediaminetetraacetic acid, pH 8.0 (Thermo Fisher Scientific), Halt protease inhibitor cocktail (1:100, Thermo Fisher Scientific), phenylmethylsulfonyl fluoride (Cell Signaling), and phosphatase inhibitor cocktail (1:100, Cell Signaling), and sonicated until completely homogenized. The samples were then centrifuged for 10 min at 10,000 g and 4°C. The supernatant was collected, further diluted at 1:10 in T-Per buffer dilution, and total protein was quantified by the Bradford assay using Pierce Coomassie Plus Assay Kit (Thermo Fisher Scientific). The relative levels of specific proteins in each brain protein extract were evaluated by western blot. The volume corresponding to 10 μg of total protein was calculated for each sample and mixed with loading buffer, prepared with Laemmli Sample Buffer (Bio-Rad Laboratories) and β-Mercaptoethanol (Sigma-Aldrich). Then, 20 μL of protein mixed with loading buffer were placed on a heat block at 100ºC for 5 min, and then loaded into Mini-PROTEAN TGX gels (Bio-Rad Laboratories) placed inside a Mini-PROTEAN Tetra Cell electrophoresis system (Bio-Rad Laboratories). After filling the electrophoresis system with running buffer (prepared using concentrated Tris-Glycine SDS buffer, Bio-Rad Laboratories), one well was loaded with 7 μL of Precision Plus Protein Kaleidoscope Pre-stained Protein Standards (Bio-Rad Laboratories), and samples were run at 160 mV for 45 min. Prior to the transfer, the PVDF membrane was activated with methanol. The gel was retrieved from the cassette, placed between premade transfer stacks (Trans-Blot Turbo Mini 0.2 µm PVDF transfer pack, Bio-Rad Laboratories) in contact with the activated PVDF membrane, and the proteins were transferred using the Trans-Blot Turbo transfer system (Bio-Rad Laboratories) at the mixed molecular weight setting. The membrane with the transferred proteins was blocked in Tris-buffered saline (TBS; prepared from a 10x TBS stock, Bio-Rad Laboratories) containing 0.1% Tween 20 (Bio-Rad Laboratories; TBS-T) and 5% BSA for 1 hour at RT with agitation. The membranes were then incubated in TBS-T 5% BSA containing antibodies against MBP (1:1000, Abcam), caspase-3 (DIL, Cell Signaling), SOD2 (DIL, Santa Cruz Biotechnology), β-actin (1:1000, Cell Signaling), or α-tubulin (DIL, company). Membranes were incubated overnight at 4°C with agitation. The following day, the membranes were washed 5 times with TBS-T and incubated with TBS-T 5% BSA containing anti-Rat IgG2a H&L (horseradish peroxidase) (1:1000, Abcam) and anti-mouse (horseradish peroxidase) (1:1000, Cell signaling) for 1 hour at RT with agitation. After 5 washes with TBS-T the membrane was incubated with Pierce ECL Western Blotting Substrate (prepared according to the manufacturer’s instructions, Thermo Fisher Scientific) for 4 min, and placed in a clear cover sheet and images of the protein bands were acquired using a ChemiDoc MP Imaging System (Bio-Rad Laboratories). The FIJI software was used to process the images acquired in the ChemiDoc, and to obtain the band intensity values for each protein of interest in each sample. Briefly, identical ROIs were used to obtain the intensity values for each protein lane (in each sample) in the different blot images. Relative protein content was calculated by dividing the intensity (arbitrary units) of the target protein by the intensity of the corresponding internal control protein (normalized to β-actin or α-tubulin).

RNAscope

Brain sections were cut at a thickness of 25 μm in the cryostat (Leica CM 3050 S), collected onto SuperFrost slides and kept at –20ºC until further staining using the Target Probes *Flt4*, *Kdr*, and *Pdgfra*, all from Advanced Cell Diagnostics, and the RNAscope® Multiplex Fluorescent Reagent Kit v2 according to all the manufacturer’s instructions, except for the target retrieval step, which was not performed.

Brain tissue lipidomics

After transcardial perfusion and euthanasia, one hemi-forebrain per mouse was dissected, weighted, snap-frozen in dry-ice, and kept at –80ºC. Samples were shipped in dry ice to the Biomarkers Core Laboratory of the Irving Institute for Clinical and Translational Research, Columbia University Irving Medical Center, for lipid extraction and targeted lipidomics by liquid chromatography–mass spectrometry, according to the Core’s established service protocols. For pairwise group comparisons of lipid expression, choice between Wilcoxon’s test or t-test was made after normality testing with Shapiro Wilk’s test. Fold-change was calculated with mean if normality was considered to be a good approximation. Alternatively, the median was used to calculate fold-change. Volcano-plots were constructed using the log2 of the fold-change on the x-axis and the negative log10 for p-values on the y-axis. For heatmap construction, z-scores were calculated compared to the control condition’s mean and standard deviation.

Murine forebrain scRNA-seq

After transcardial perfusion and euthanasia, individual hemi-forebrains (one per mouse) were dissected, groups of two hemi-forebrains (from two different mice) were pooled, and dissociated using the Adult Brain Dissociation Kit (Miltenyi Biotec) according to the manufacturer's instructions. After cell counting, approximately 10,000 cells per sample were loaded onto the Chromium Single Cell A Chip and run on a 10x Genomics Chromium Controller. Sequencing libraries were generated using the Chromium Single Cell 3' Library & Gel Bead Kit v2 (PN-1000190, 10x Genomics). After a cDNA library quality control step performed on an Agilent DNA 7500 system, libraries were sequenced on the Illumina NovaSeq 6000, using a NovaDeq S4 PE100 flow cell (paired-end reads). The single-cell sequence pre-processing was performed using the standard 10x Genomics Cell Ranger Single Cell Software Suite (v.6.1.1). Briefly, raw sequencing data were demultiplexed, aligned to the mouse genome mm10, and the reads aligned to each gene were counted. Cell filtration, normalization, clustering and differential expression analyses were performed using standard Seurat package procedures (v.4.0.5). For quality assurance, cells that had unique gene counts over 4,000 or less than 200, and >25% mitochondrial content were removed. SCTransform was used to normalize and scale data, and to remove confounding sources of variations from sequencing depth and mitochondria mapping percentage. All samples were integrated, and canonical correlation analysis was performed to identify common sources of variation among samples and to remove batch effects. The first 30 canonical correlation vectors were used for subsequent clustering analysis and visualization using Uniform Manifold Approximation and Projection (UMAP). Function "FindAllMarkers" was used to identify cluster-specific marker genes. Cell clusters were manually annotated as cell types based on literature. Cell doublets that expressed more than one cell type markers were excluded from downstream analyses. None-ribosomal protein genes were tested for differential expression between the VEGF-C/D trap and control groups within each cluster and each cell type using the MAST model. Genes with adjusted $p < 0.05$ and average log2 fold-change > 0.25 were defined as differentially expressed genes (DEGs). Pathway analysis of DEGs from each cluster was performed using Metascape. Pathways with $-\log10(p) > 2$ ($p < 0.01$) were considered significantly altered.

Brain tissue proteomics

For proteomics analysis, one hemi-forebrain per mouse was dissected, weighted, snap-frozen in dry-ice and kept at –80°C until protein extraction. Protein extraction and quantification was performed using the methodology previously described for western blot analysis. All samples were diluted to the same final concentration in Lysis buffer before shipment. Brain protein levels were measured using the proximity extension immunoassay technology – Olink Target 48 Mouse Cytokine Panel via the services of Vanderbilt University Medical Center High-

Throughput Biomarker Core. Data were received as concentration values in pg/mL which were used to calculate the fold change relative to the control group average. The value of zero was attributed to samples for which a concentration was below the detection limit. Proteins for which one or more sample values deviated more than 4 standard deviations, relative to the average of all samples, were excluded from the analysis.

Flow cytometry

After mice perfusion with ice-cold 1x PBS with heparin, the ventral side of the neck was exposed and two superficial cervical lymph nodes (one from each side) were pooled together with all deep cervical lymph nodes, in ice-cold Roswell Park Memorial Institute 1640 (RPMI 1640, Genesee Scientific). Individual meninges were immediately dissected from the mouse's skull cap, in ice-cold RPMI 1640. All tissues were digested for 25 min at 39°C with 1 mg/mL of Collagenase VIII, 1 mg/mL of Collagenase D and 50 U/mL of DNAse I (all from Sigma-Aldrich) in RPMI 1640. The same volume of RPMI 1640 with 10% fetal bovine serum (Corning) was added to the digested tissue, which was then filtered through a 70 μm cell strainer (Fisher Scientific). The cell pellets were washed and resuspended in ice-cold fluorescence-activated cell sorting (FACS) buffer (pH 7.4, 0.1 M PBS; 1 mM EDTA and 1% BSA). For extracellular staining, cells were preincubated for 10 min at 4°C with Fc-receptor blocking solution (anti-CD16/32, clone 93, Biolegend, 1:200 in FACS buffer) and then incubated for 20 min at 4°C with different combinations of the following antibodies anti-CD45-PerCP-Cy5.5, clone 30-F11 (1:200, BD Biosciences), CD19-FITC (1:200, Biolegend), CD19-APC-Cy7 (1:100, Biolegend), TCRβ-BV711 (1:100, BD Biosciences), CD4-PE-Dazzle 594 (1:200, Biolegend), CD4-PE-Cy7 (1:300, Biolegend), CD8α-PB (1:200, BD Biosciences). Cell viability was determined by using Zombie AQUA (ZA) Viability dye (Biolegend) following the manufacturer's instructions. Fluorescently labeled single-cell suspensions were washed with FACS buffer and acquired in an Attune NxT Flow Cytometer (Thermo Fisher Scientific) coupled to Attune NxT Autosampler. Data analysis was performed using FlowJo software (BD Biosciences). Briefly, singlets were gated in FSC-H *vs*. FSC-A, leukocytes were gated in SSC-A *vs*. FSC-A, followed by the gating of live leukocytes as $CD45^{+}ZA^{-}$. Cells were then gated for the appropriate cell type markers.

ELISA for human VEGF-C

The Human VEGF-C Quantikine ELISA Kit (cat# DVEC00, R&D systems) was used to quantify VEGF-C protein levels in human plasma and CSF samples, following the manufacturer’s instructions. Briefly, 100 μL of assay diluent RD1W was added to each well, followed by 50 μL of standard or sample. Per the instructions, the Calibrator Diluent RD6U was pre-diluted 1:2 for plasma samples and corresponding standards, and 1:5 for CSF and corresponding standards. Then, plasma and CSF samples were diluted in each corresponding pre-diluted Calibrator Diluent RD6U at 1:2. After incubating for two hours at RT with agitation, the plate was washed 4 times with 400 μL of wash buffer, followed by incubation with 200 μL of human VEGF-C conjugate for two hours at RT with agitation. A washing step was performed as previously, and 200 μL of substrate solution were added to each well and incubated for 30 min at RT, covered from light and without agitation. The reaction was stopped by adding 50 μL of stop solution to each well. The plates were read immediately on Fluorstar Omega (BMG Labtech) by measuring the optical density at 450 nm, with a 540 nm wavelength correction. VEGF-C concentrations were calculated based on the standard curve and the sample dilution factor.

QUANTIFICATIONS AND STATISTICAL ANALYSES

Sample sizes for each experiment were appropriately chosen on the basis of standard power calculations (with $\alpha = 0.05$ and power of 0.8) performed for similar experiments that were previously published by the authors. All quantifications and analyses were performed by a blinded experimenter. Microsoft Excel was used to record values and perform calculations in each experiment and all the statistical analyses were performed using Prism 9. Two outliers were identified and excluded in two different experiments using the Rout test (Q = 1%): one in the data presented in Figures S6B, S6I, S6K (from the 2-week VEGF-C/D trap group), and another in the data presented in Figures 3C, 3E–3G, S4F–S4H (male from the *Cdh5*$^{CreERT2/+}$*;Prox1*$^{flox/flox}$ group). The Kolmogorov-Smirnov or Shapiro-Wilk tests were used to evaluate data distribution. Data in graphs are presented as mean ± standard error mean (SEM). Two-tailed unpaired Student’s T test was used to compare two groups. Two-way ANOVA with Sidak’s multiple comparisons or uncorrected Fisher’s LSD test were used to analyze data involving multiple groups and two independent variables. Correlation analyses were performed using the Pearson r. Statistical tests used in scRNA-seq and lipidomics analyses are specified in the respective methodological sections. Statistical significance was considered for p-values < 0.05. The exact p-values < 0.05 are discriminated in all graphs.

Figure legends

Figure 1. Decreased VEGF-C/D signaling and meningeal lymphatic vessel regression lead to loss of MOLs and defects in brain lipid composition.

(A) Eight-week-old C57BL/6J male mice received 2 or 3 injections (syringe icons) of AAV9-*mVegfr3*$_{4–7}$*-Ig* (control) or AAV9-*mVegfr3*$_{1–3}$*-Ig* (VEGF-C/D trap), and tissues were collected at 5 and 7 weeks after the first injection (mouse icons). AAV9, adeno-associated virus serotype 9; *mVegfr3*, murine vascular endothelial growth factor receptor 3 gene; VEGF-C/D, vascular endothelial growth factor C/D.

(B and C) Representative images of meningeal dural whole mounts stained for lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1, green) and 4,6-diamidino-2-phenylindole (DAPI, blue) in (B), and quantifications of lymphatic vessel length per area of region of interest (ROI) at each time point in (C).

(D and E) Representative images of the staining for myelin basic protein (MBP, magenta) in brain coronal sections showing the ROI outlining the corpus callosum in (D), and quantifications of MBP signal coverage within the ROI in (E).

(F and G) Western blots showing the bands relative to MBP isoforms and beta-actin (β-actin) in forebrain protein samples in (F), and the corresponding quantifications of MBP (normalized to β-actin) presented as fold change relative to the control group in (G).

(H and I) Representative images of quaking 7 (with antibody clone CC1, red), platelet derived growth factor receptor alpha (PDGFRα, green), and DAPI (blue) stainings in the corpus callosum in (H), and quantifications of the number of CC1^{+}PDGFRα^{-} cells per area in (I).

(J and K) Representative images of the CC1 (red), for PDGFRα (green), and DAPI (blue) stainings in the motor cortex in (J), and quantifications of the number of CC1^{+}PDGFRα^{-} cells per area in (K).

(L) Heatmap representing the relative expression levels (z-score in scale bar) of significantly altered lipid species in the forebrains of mice from the VEGF-C/D trap group compared to the control group at 7 weeks.

Data in (C), (E), (G), (I), and (K) are presented as mean ± standard error mean (SEM); data in (L) are presented as change versus the control group; n = 10–12 mice per group in (C); n = 12 mice per group in (E); n = 5 mice per group in (G); n = 12–16 mice per group in (I) and (K), data in each graph were pooled from 2 out of 3 independent experiments; n = 4 mice per group in (L); all experiments involved male mice only; two-way ANOVA with Sidak's multiple comparisons test between control and VEGF-C/D trap groups in (C), (E), (I) and (K); two-tailed unpaired Student's t-test in (G); two-tailed unpaired Student's t-test or Wilcoxon rank sum test were used according to data normality as assessed using the Shapiro-Wilk test in (L).

See also Figure S1 and Supplementary File 1.

Figure 2. Altered gene expression in MOLs precedes cell loss in mice with ablated meningeal lymphatics.

(A) Eight-week-old C57BL/6J male mice received 2 injections (syringe icons) of AAV9-*mVegfr3*$_{4–7}$*-Ig* (control) or AAV9-*mVegfr3*$_{1–3}$*-Ig* (VEGF-C/D trap), and forebrain tissues were collected 5 weeks later (mouse icon). The forebrain tissues were processed for single-cell RNA sequencing (scRNA-seq) using 10x Genomics reagents and equipment.

(B) Uniform manifold approximation and projection (UMAP) representation of the 21 clusters and respective cluster annotation. Oligodendrocyte precursor cells (OPCs), newly-formed oligodendrocytes (NFOLs), myelinating oligodendrocytes (myelinating OLs), mature oligodendrocytes (MOLs), border-associated macrophages (BAMs), blood endothelial cells (BECs), vascular smooth muscle cells (vSMCs), and choroid plexus (CP) epithelial cells.

(C) Total number of significantly down- or up-regulated genes (adjusted p-value < 0.05) per cluster, in the VEGF-C/D trap group versus (*vs.*) the control group.

(D) Heatmap showing relative expression levels (scale bar) of all differentially expressed genes (DEGs) in the MOLs 2 cluster.

(?) Graph showing the altered functional pathways in the MOLs 2 cluster of the VEGF-C/D trap group for a –log10(*P value*) > 2 (compared to the control group).

(? and ?) Western blot showing the bands relative to superoxide dismutase 2 (SOD2) and alpha-tubulin (α-tubulin) in forebrain protein samples at 7 weeks in (F), and the corresponding quantifications of SOD2 (normalized to α-tubulin) presented as fold change relative to the control group in (G).

Data in (?) are presented as mean ± SEM; n = 5 mice per group; experiments involved male mice only; two-tailed unpaired Student’s t-test.

See also Figures S2 and S3.

Figure 3. A genetic model of impaired meningeal lymphatic function presents concomitant demyelination and altered brain-associated adaptive immunity.

(A) Littermate male and female *Cdh5*$^{CreERT2/+}$ and *Cdh5*$^{CreERT2/+}$*;Prox1*$^{flox/flox}$ mice received 3 consecutive daily injections of tamoxifen (50 mg/kg, syringe icons) at 8–13 weeks of age. The injections were repeated at week 1 and tissues were collected at week 3 (mouse icon).

(B and C) Representative images of meningeal dural whole mounts stained for LYVE-1 (green) and DAPI (blue) in (B), and quantifications of lymphatic vessel length per ROI in (C).

(D–G) Representative images of CC1 (red), PDGFRα (green), and DAPI (blue) stainings in the motor cortex in (D), quantifications of CC1^{+}PDGFRα^{-} cells per area in (E), quantifications of PDGFRα^{+} cells per area in (F), and graph showing the correlation between the density of CC1^{+}PDGFRα^{-} cells in the motor cortex (MC) and the length of meningeal lymphatic vessels in (G).

(H–K) Representative images of transmission electron microscopy in the corpus callosum in (H), and quantifications of myelin sheet G-ratio in (I), percentage of myelinated axons in (J) and total number of axons per image in (K).

(L and M) Representative images of cluster of differentiation 3 (CD3, red) and DAPI (blue) stainings in the leptomeninges in (L), and quantifications of CD3^{+} cells per area of leptomeningeal tissue in (M).

(N–T) Representative flow cytometry dot plots of cervical lymph node leukocytes in (N), frequencies (within live CD45^{+} cells) of B cells in (O), CD4^{+} T cells in (P) and CD8^{+} T cells in (Q), and total cell numbers of B cells in (R), CD4^{+} T cells in (S) and CD8^{+} T cells in (T).

Data in (C), (E), (F), (I–K), (M) and (O–T) are presented as mean ± SEM; n = 13–14 mice per group in (C), (E), (F) and (G), pooled from two independent experiments (6 males and 8 females in the *Cdh5*$^{CreERT2/+}$ group; 7 males and 6 females in the *Cdh5*$^{CreERT2/+}$*;Prox1*$^{flox/flox}$ group); n = 5 mice per group (all males) in (I–K); n = 8 mice per group (3 males and 5 females in the *Cdh5*$^{CreERT2/+}$ group; 5 males and 3 females in the *Cdh5*$^{CreERT2/+}$*;Prox1*$^{flox/flox}$ group) in (M); n = 6 mice per group (all males) in (O–T); two-tailed unpaired Student’s t-test was used in (C), (E), (F), (I–K), (M), and (O–T); data in (G) resulted from a Pearson correlation.

See also Figure S4.

Figure 4. The loss of MOLs observed upon meningeal lymphatic regression is abrogated in immunodeficient mice.

(A) Littermate recombination activating gene 2 deficient (*Rag2*$^{-/-}$) male mice received 3 injections (syringe icons) of AAV9-*mVegfr3*$_{4-7}$-*Ig* (control) or AAV9-*mVegfr3*$_{1-3}$-*Ig* (VEGF-C/D trap) at 9–15 weeks of age, and tissues were collected 7 weeks after the first injection (mouse icon).

(B and C) Representative images of meningeal dural whole mounts stained for LYVE-1 (green) and DAPI (blue) in (B), and quantifications of lymphatic vessel length per area of ROI in (C).

(D and E) Western blot showing the bands relative to SOD2 and α-tubulin in forebrain protein samples in (D), and the corresponding quantifications of SOD2 (normalized to α-tubulin) presented as fold change relative to the control group in (E).

(F and G) Representative images of the staining for MBP (magenta) in brain coronal sections showing the ROI outlining the corpus callosum in (F), and quantifications of MBP signal coverage within the ROI in (G).

(H and I) Representative images of CC1 (red), PDGFRα (green), and DAPI (blue) stainings in the motor cortex in (H), and quantifications of the number of CC1^{+}PDGFRα^{-} cells per area in (I).

(J) Eight-week-old C57BL/6J male mice received 3 injections (syringe icons) of AAV9-*mVegfr3*$_{4-7}$-*Ig* (control) or AAV9-*mVegfr3*$_{1-3}$-*Ig* (VEGF-C/D trap). At week 3, all mice started a diet containing PLX5622 (600 parts per million) and tissues were collected 4 weeks later (mouse icon).

(K and L) Representative images of meningeal dural whole mounts stained for LYVE-1 (green) and DAPI (blue) in (K), and quantifications of lymphatic vessel length per area of ROI in (L).

(M and N) Western blot showing the bands relative to SOD2 and α-tubulin in forebrain protein samples in (M), and the corresponding quantifications of SOD2 (normalized to α-tubulin) presented as fold change relative to the control group in (N).

(O and P) Representative images of the staining for MBP (magenta) in brain coronal sections showing the ROI outlining the corpus callosum in (O), and quantifications of MBP signal coverage within the ROI in (P).

(Q and R) Representative images of CC1 (red), PDGFRα (green), and DAPI (blue) stainings in the motor cortex in (Q), and quantifications of the number of CC1^{+}PDGFRα^{-} cells per area in (R).

Data in (C), (E), (G), (I), (L), (N), (P) and (R) are presented as mean ± SEM; n = 9–13 mice per group in (C) and (I); n = 6 mice per group in (E); n = 12 mice per group in (G); n = 13–14 mice per group in (L) and (R); n = 5 mice per group in (N); n = 10 mice per group in (P); data in (C), (G), (I), (L), (P) and (R) were pooled from two independent experiments; all experiments involved male mice only; two-tailed unpaired Student's t-test.

See also Figure S5.

Figure 5. Delayed brain remyelination due to meningeal lymphatic dysfunction is linked to immunosuppression and is only observed in immunocompetent mice.

(A) Eight-week-old C57BL/6J male mice received 3 injections (syringe icons) of AAV9-*mVegfr3*$_{4–7}$*-Ig* (control) or AAV9-*mVegfr3*$_{1–3}$*-Ig* (VEGF-C/D trap). Mice from both groups were exposed to a diet containing 0.2% cuprizone for a total of 4 weeks and then returned to standard diet (week 0). Tissues were collected at 2 or 4 weeks of remyelination (mouse icons).

(B and C) Representative images of the staining for MBP (magenta) in brain coronal sections showing the ROI outlining the corpus callosum in (B), and quantifications of MBP signal coverage in the corpus callosum (C).

(D and E) Representative images of CC1 (red), PDGFRα (green), and DAPI (blue) stainings in the corpus callosum in (D), and quantifications of the number of CC1^{+}PDGFRα^{-} cells per area in (E).

(F–I) Representative images of transmission electron microscopy in the corpus callosum at 2 weeks in (F), and quantifications of myelin sheet G-ratio in (G), percentage of myelinated axons in (H) and total number of axons per image in (I).

(J and K) Western blots showing the bands relative to caspase-3 and β-actin in forebrain protein samples at 2 weeks in (J), and the corresponding quantifications of caspase-3 (normalized to β-actin) presented as fold change relative to the control group in (K).

(L) Wild type and *Rag2*$^{-/-}$ male mice received 3 injections (syringe icons) of AAV9-*mVegfr3*$_{4–7}$*-Ig* (control) or AAV9-*mVegfr3*$_{1–3}$*-Ig* (VEGF-C/D trap) at 8–9 weeks of age. Mice from all groups were exposed to a diet containing 0.2% cuprizone for a total of 4 weeks and then returned to regular chow (week 0). Tissues were collected at 2 weeks of remyelination (mouse icon).

(M and N) Representative images of CC1 (red), oligodendrocyte transcription factor 2 (OLIG2, green), PDGFRα (grey), and DAPI (blue) stainings in the corpus callosum in (M), and quantifications of the number of CC1^{+}OLIG2^{+}PDGFRα^{-} cells per area in (N).

(O) Heatmap showing the relative expression levels of immune-related proteins in the forebrains of mice from each group (scale bar shows the fold change relative to wild type control group).

Data in (C), (E), (G–I), (K) and (N) are presented as mean ± SEM; data in (O) are presented as change versus the wild type control group; n = 7–9 mice per group in (C), representative of 2 independent experiments; n = 14–15 mice per group in (E), pooled from 2 independent experiments; n = 5 mice per group in (G–I); n = 4 mice per group in (K); n = 5–6 mice per group in (N) and (O); all experiments involved male mice only; two-way ANOVA with Sidak's multiple comparisons test between control and VEGF-C/D trap groups in (C) and (E); two-tailed unpaired Student’s t-test in (G–I) and (K); two-way ANOVA with uncorrected Fisher’s LSD test for comparisons between rows and columns (wild type control *vs*. *Rag2*$^{-/-}$ control; wild type VEGF-C/D trap *vs*. *Rag2*$^{-/-}$ VEGF-C/D trap; wild type control *vs*. wild type VEGF-C/D trap; and *Rag2*$^{-/-}$ control *vs*. *Rag2*$^{-/-}$ VEGF-C/D trap) in (N) and (O).

See also Figure S5, Figure S6 and Supplementary File 1.

Figure 6. Multiple sclerosis patients present lower levels of VEGF-C in the cerebrospinal fluid shortly upon relapses.

(A and B) Graphs showing the levels of VEGF-C in plasma samples in (A), and in cerebrospinal fluid (CSF) samples in (B), from healthy controls and multiple sclerosis (MS) patients.

(C) Graph showing the comparison between VEGF-C levels in the CSF of MS patients that relapsed within 60 days (< 60) or more than 60 days (> 60) before sample collection.

Data in (A–C) are presented as mean ± SEM; n = 35 healthy controls and n = 29 MS patients in (A); n = 36 healthy controls and n = 33 MS patients in (B); n = 13 MS patients in the < 60 group, n = 20 MS patients in the > 60 group in (C); two-tailed Mann Whitney test.

See also Figure S6.

Supplemental information

Meningeal lymphatic drainage regulates oligodendrocyte survival and brain myelination

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Figure S1, related to Figure 1. Decreased VEGF-C/D signaling affects meningeal, but not peripheral, lymphatic vessels.

(A) Eight-week-old C57BL/6J male mice received a single injection (syringe icon) of AAV9-*mVegfr3*$_{4–7}$*-Ig* (control) or AAV9-*mVegfr3*$_{1–3}$*-Ig* (VEGF-C/D trap), and tissues were collected 5 weeks later (mouse icon). AAV9, adeno-associated virus serotype 9; VEGF-C/D, vascular endothelial growth factor C/D; VEGFR3, vascular endothelial growth factor receptor 3.

(B–D) Representative images of meningeal dural whole mounts stained for lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1, green) and cluster of differentiation 31 (CD31, red) in (B), and quantifications of lymphatic vessel length per area of region of interest (ROI) in (C) and of CD31$^+$ blood vessel coverage in (D).

(E and F) Representative image of ear skin whole mount stained for LYVE-1 (green) and 4,6-diamidino-2-phenylindole (DAPI, blue) in (E), and quantifications of total lymphatic vessel length in (F).

(G and H) Representative image of a transverse cross section of small intestine stained for LYVE-1 (green) and DAPI (blue) in (G), and quantifications of lymphatic vessel coverage (as % of the DAPI area) in (H).

(I and J) Representative images of the staining for myelin basic protein (MBP, magenta) in brain coronal sections showing the ROI depicting the motor cortex in (I), and quantifications of MBP signal coverage within the ROI in (J).

(K) Volcano plot depicting all the analyzed lipid classes in the forebrain of mice from the different groups and highlighting (in blue) the significantly altered lipid classes in the VEGF-C/D trap group compared to the control group at 7 weeks.

Data in (C), (D), (F), (H) and (J) are presented as mean ± SEM; n = 4–5 mice per group in (C), (D) and (H); n = 4 mice per group in (F) and (K); n = 12 mice per group in (J); all experiments involved male mice only; two-tailed unpaired Student’s t-test in (C), (D), (F) and (H); two-way ANOVA with Sidak's multiple comparisons test between control and VEGF-C/D trap groups in (J); two-tailed unpaired Student’s t-test or Wilcoxon rank sum test were used according to data normality as assessed using the Shapiro-Wilk test in (K).

See also Supplementary File 1.

Figure S2, related to Figure 2. Single-cell transcriptomic profiling of forebrain cells in mice with ablated meningeal lymphatics at 5 weeks reveals a higher number of differentially expressed genes in MOLs and astrocytes.

(A) Stacked violin plots depicting the expression levels of genes considered for the annotation of the 21 forebrain cell clusters. Oligodendrocyte precursor cells (OPCs), newly-formed oligodendrocytes (NFOLs), myelinating oligodendrocytes (myelinating OLs), mature oligodendrocytes (MOLs), border-associated macrophages (BAMs), blood endothelial cells (BECs), vascular smooth muscle cells (vSMCs), and choroid plexus (CP) epithelial cells.

(B and C) Uniform manifold approximation and projection (UMAP) representations of the 21 clusters in the control and VEGF-C/D groups in (B), and pie charts showing similar proportions of the 21 clusters (within all brain cells) in each group (C).

(D) Graph showing the altered functional pathways in the astrocytes 2 cluster of the VEGF-C/D trap group for a –log10(*P value*) > 2 (compared to the control group).

(E and F) Representative images of glial fibrillary acidic protein (GFAP, magenta) and DAPI (blue) stainings in the corpus callosum of mice in (E), and quantifications of GFAP coverage in (F).

(G–I) Representative images of CD68 (red), ionized calcium-binding adaptor molecule 1 (IBA1, green), and DAPI (blue) stainings in the corpus callosum in (G), and quantifications of the number of IBA1$^+$ cells per area in (H) and of CD68$^+$ area per total number of IBA1$^+$ cells (i.e., CD68 expression per IBA1$^+$cell) in (I).

Data in (F), (H) and (I) are presented as mean ± SEM; n = 10 mice per group; all experiments involved male mice only; two-tailed unpaired Student’s t-test.

Figure S3, related to Figure 2. Number and proliferation of OPCs is not affected by the reduction in VEGF-C/D signaling and meningeal lymphatic vessel ablation.

(A) UMAP representations illustrating the expression levels of *Flt4, Kdr, Ccbe1*, *Vegfc*, and *Vegfd* in the different forebrain cell clusters. Scale bar represents relative expression levels.

(B) Heatmap showing the relative expression levels (scale bar) of the genes *Flt4, Kdr*, *Ccbe1*, *Vegfc*, and *Vegfd* in different forebrain glial and immune cell clusters.

(C–E) Representative image of the staining for *Pdgfra* (red), *Kdr* (green), and *Flt4* (light grey) mRNA transcripts by RNAscope, alongside DAPI (blue), in the corpus callosum in (C), frequencies of *Kdr*$^+$ cells within total *Pdgfra*$^+$ cells in (D), and numbers of total *Pdgfra*$^+$ cells per area in (E).

(F–H) Representative image of the staining for *Pdgfra* (red), *Kdr* (green), and *Flt4* (light grey) mRNA transcripts by RNAscope, alongside DAPI (blue), in the motor cortex in (F), frequencies of *Kdr*$^+$ cells within total *Pdgfra*$^+$ cells in (G), and numbers of total *Pdgfra*$^+$ cells per area in (H).

(I) Eight-week-old C57BL/6J male mice received two or three injections (light grey syringe icons) of AAV9-*mVegfr3*$_{4\text{–}7}$*-Ig* (control) or AAV9-*mVegfr3*$_{1\text{–}3}$*-Ig* (VEGF-C/D trap). Mice received 3 consecutive daily injections of 5-ethynyl-2-deoxyuridine (EdU; black syringe icons), and tissues were collected 5 and 7 weeks (mouse icons) after the initial injections with the AAVs.

(J–L) Representative image of nerve/glial antigen 2 (NG2, green), platelet derived growth factor receptor alpha (PDGFRα, red), EdU (light grey), and DAPI (blue) stainings in the corpus callosum in (J), numbers of NG2$^+$PDGFRα$^+$ cells per area in (K) and frequencies of EdU$^+$ cells within total NG2$^+$PDGFRα$^+$ cells (dividing OPCs) in (L).

(M–O) Representative image of NG2 (green), PDGFRα (red), EdU (light grey), and DAPI (blue) stainings in the motor cortex in (M), numbers of NG2$^+$PDGFRα$^+$ cells per area in (N) and frequencies of EdU$^+$ cells within total NG2$^+$PDGFRα$^+$ cells (dividing OPCs) in (O).

(P) Eight-week-old C57BL/6J male mice received weekly injections (syringe icons; a total of 7 injections) of anti-VEGFR2 antibodies or isotype-matched control antibodies (100 μg of each antibody per injection), and tissues were collected at week 7 (mouse icon).

(Q and R) Representative images of meningeal dural whole mounts stained for LYVE-1 (green) and DAPI (blue) in (Q), and quantifications of lymphatic vessel length per area of ROI in each group in (R).

(S–U) Representative images of CC1 (red), PDGFRα (green), and DAPI (blue) stainings in the corpus callosum in (S), and numbers of PDGFRα^+ cells per area in (T), and of CC1$^+$PDGFRα^- cells per area in (U).

(V–X) Representative images of CC1 (red), PDGFRα (green), and DAPI (blue) stainings in the motor cortex in (V), and numbers of PDGFRα^+ cells per area in (W), and of CC1$^+$PDGFRα^- cells per area in (X).

Data in (D), (E), (G), (H), (K), (L), (N), (O), (R), (T), (U), (W) and (X) are presented as mean ± SEM; n = 5–6 mice per group in (D), (E), (G) and (H); n = 5 mice per group in (K), (L), (N) and (O); n = 6–7 mice per group in (R), (T), (U), (W) and (X); data in (K) and (N) are representative of 2 independent experiments; all experiments involved male mice only; two-way ANOVA with Sidak's multiple comparisons test between control and VEGF-C/D trap groups in (D), (E), (G), (H), (K), (L), (N) and (O); two-tailed unpaired Student’s t-test in (R), (T), (U), (W) and (X).

Figure S4, related to Figure 3. Mice with impaired meningeal lymphatic vessels seem to present higher recruitment of adaptive immune cells to the brain borders.

(A and B) Representative images of meningeal dural whole mounts stained for CD31 (red) and DAPI (blue) in (A), and quantifications of blood vessel coverage within the dural ROI in the *Cdh5*$^{CreERT2/+}$ and *Cdh5*$^{CreERT2/+}$*;Prox1*$^{flox/flox}$ groups in (B).

(C and D) Representative images of ear skin whole mounts stained for LYVE-1 (green) and DAPI (blue) in (C), and quantifications of total lymphatic length in the *Cdh5*$^{CreERT2/+}$ and *Cdh5*$^{CreERT2/+}$*;Prox1*$^{flox/flox}$ groups in (D).

(E–H) Representative images of CC1 (red), PDGFRα (green), and DAPI (blue) stainings in the motor cortex in (E), quantifications of CC1^{+}PDGFRα^{-} cells per area in (F), quantifications of PDGFRα^{+} cells per area in (G), and graph showing the correlation between the density of CC1^{+}PDGFRα^{-} cells in the corpus callosum (CC) and the length of meningeal lymphatic vessels in (H).

(I–K) Representative images of CD68 (red), IBA1 (green), and DAPI (blue) stainings in the corpus callosum in (I), and quantifications of the number of IBA1^{+} cells per area in (J) and of CD68^{+} area per total number of IBA1^{+} cells (i.e., CD68 expression per IBA1^{+}cell) in (K).

(L–N) Representative images of CD68 (red), IBA1 (green), and DAPI (blue) stainings in the motor cortex in (L), and quantifications of the number of IBA1^{+} cells per area in (M) and of CD68^{+} area per total number of IBA1^{+} cells (i.e., CD68 expression per IBA1^{+}cell) in (N).

(O–R) Representative flow cytometry dot plots of meningeal dura leukocytes from *Cdh5*$^{CreERT2/+}$ and *Cdh5*$^{CreERT2/+}$*;Prox1*$^{flox/flox}$ mice in (O), total numbers of live CD45^{+} cells in (P), and frequencies (within live CD45^{+} cells) of B cells in (Q), and TCRβ^{+} T cells in (R).

(S–V) Representative flow cytometry dot plots of meningeal dura leukocytes from mice of the control or VEGF-C/D trap groups (7 weeks’ time-point) in (S), total numbers of live CD45^{+} cells in (T), and frequencies (within live CD45^{+} cells) of B cells in (U), and TCRβ^{+} T cells in (V).

Data in (B), (D), (F), (G), (J), (K) (M), (N), (P–R) and (T–V) are presented as mean ± SEM; n = 8 mice per group (3 males and 5 females in the *Cdh5*$^{CreERT2/+}$ group, 5 males and 3 females in the *Cdh5*$^{CreERT2/+}$*;Prox1*$^{flox/flox}$ group) in (B) and (D), representative of two independent experiments; n = 13–14 mice per group (6 males and 8 females in the *Cdh5*$^{CreERT2/+}$ group; 7 males and 6 females in the *Cdh5*$^{CreERT2/+}$*;Prox1*$^{flox/flox}$ group) in (F), (G) and (H); n = 10–12 mice (5 males and 5 females in the *Cdh5*$^{CreERT2/+}$ group; 9 males and 3 females in the *Cdh5*$^{CreERT2/+}$*;Prox1*$^{flox/flox}$ group) in (J), (K), (M) and (N), n = 6 male mice per group in (P–R); n = 5 male mice per group in (T–V); data in (F), (G), (H), (J), (K), (M), and (N) were pooled from two independent experiments; two-tailed unpaired Student’s t-test in (B), (D), (F), (G), (J), (K), (M), (N), (P–R), (T–V); data in (H) resulted from a Pearson correlation.

Figure S5, related to Figures 4 and 5. Meningeal lymphatic dysfunction does not induce MOL loss in the corpus callosum of immunodeficient mice, or affect the degree of cuprizone-induced demyelination.

(A and B) Representative images of CC1 (red), PDGFRα (green), and DAPI (blue) stainings in the corpus callosum of *Rag2*$^{-/-}$ male mice (in the control or VEGF-C/D trap groups) in (A), and quantifications of the number of CC1^{+}PDGFRα^{-} cells per area in (B).

(C and D) Representative images of IBA1 (red) and DAPI (blue) stainings in the cortex of C57BL/6J male mice exposed to control or PLX5622 diets in (C), and quantifications of the number of IBA1^{+} cells per area in (D).

(E and F) Representative images of CC1 (red), PDGFRα (green), and DAPI (blue) stainings in the corpus callosum of C57BL/6J male mice exposed to a PLX5622 diet (in the control or VEGF-C/D trap groups) in (E), and quantifications of the number of CC1^{+}PDGFRα^{-} cells per area in (F).

(G) Eight-week-old C57BL/6J male mice received 3 injections (syringe icons) of AAV9-*mVegfr3*$_{4\text{–}7}$*-Ig* (control) or AAV9-*mVegfr3*$_{1\text{–}3}$*-Ig* (VEGF-C/D trap). At week 1, mice from both groups started a diet containing 0.2% cuprizone, and tissues were collected at week 5 (mouse icon).

(H and I) Representative images of CC1 (red), PDGFRα (green), and DAPI (blue) stainings in the corpus callosum of C57BL/6J male mice exposed to a cuprizone diet (in the control or VEGF-C/D trap groups) in (H), and quantifications of the number of CC1^{+}PDGFRα^{-} cells per area in (I).

(J and K) Representative images of CC1 (red), PDGFRα (green), and DAPI (blue) stainings in the motor cortex of C57BL/6J male mice exposed to a cuprizone diet (in the control or VEGF-C/D trap groups) in (J), and quantifications of the number of CC1^{+}PDGFRα^{-} cells per area in (K).

Data in (B), (D), (F), (I), and (K) are presented as mean ± SEM; n = 9–13 mice per group in (A); n = 4 mice per group in (D); n = 13–14 mice per group in (F); n = 12 mice per group in (I) and (K), pooled from 2 independent experiments; all experiments involved male mice only; two-tailed unpaired Student's t-test.

Figure S6, related to Figures 5 and 6. Delayed replenishment of MOLs in mice with ablated meningeal lymphatics is not linked to altered brain OPC, astrocyte or innate immune responses.

(A and B) Representative images of meningeal dural whole mounts stained for LYVE-1 (green) and DAPI (blue) in (A), and quantifications of lymphatic vessel length per area of ROI in (B).

(C and D) Representative images of the staining for myelin basic protein (MBP) in brain coronal sections showing the ROI depicting the motor cortex in (C), and quantifications of MBP signal coverage within the ROI in (D).

(E and F) Representative images of CC1 (red), PDGFRα (green), and DAPI (blue) stainings in the motor cortex in (E), and quantifications of the number of CC1$^+$PDGFRα$^-$ cells per area in (F).

(G) Heatmap representing the relative expression levels (z-score in scale bar) of significantly altered lipid species in the forebrains of mice from the VEGF-C/D trap group compared to the control group at 2 weeks.

(H and I) Representative images of NG2 (green), PDGFRα (red), and DAPI (blue) stainings in the corpus callosum in (H), and quantifications of the number of NG2$^+$PDGFRα$^+$ cells per area in (I).

(J and K) Representative images of NG2 (green), PDGFRα (red), and DAPI (blue) stainings in the motor cortex in (J), and quantifications of the number of NG2$^+$PDGFRα$^+$ cells per area in (K).

(L and M) Representative images of GFAP (magenta) and DAPI (blue) stainings in the corpus callosum of mice at 2 weeks in (L), and quantifications of GFAP coverage in (M).

(N–P) Representative images of CD68 (red), IBA1 (green), and DAPI (blue) stainings in the corpus callosum at 2 weeks in (N), and quantifications of the number of IBA1$^+$ cells per area in (O) and of CD68$^+$ area per total number of IBA1$^+$ cells (i.e., CD68 expression per IBA1$^+$cell) in (P).

(Q) Eight-week-old C57BL/6J male mice received 3 injections (syringe icons) of AAV9-*mVegfr3*$_{4–7}$*-Ig* (control) or AAV9-*mVegfr3*$_{1–3}$*-Ig* (VEGF-C/D trap). Mice from both groups were exposed to a diet containing 0.2% cuprizone for a total of 4 weeks and then were switched to a PLX5622 diet (week 0). Tissues were collected at 2 weeks of remyelination (mouse icon).

(R and S) Representative images of CC1 (red), oligodendrocyte transcription factor 2 (OLIG2, green), PDGFRα (grey), and DAPI (blue) stainings in the corpus callosum in (R), and quantifications of the number of CC1$^+$OLIG2$^+$PDGFRα^- cells per area in (S).

(T) Heatmap showing the relative expression levels of immune-related proteins in the forebrains of mice from each group at 2 weeks (scale bar shows the fold change relative to the control group).

(U) Table showing the results of the simple linear and multiple linear (controlled for age, sex and interaction between sex and diagnosis) regression analyses relative to the effect of diagnosis on cerebrospinal fluid (CSF) VEGF-C levels in healthy controls (HC) and multiple sclerosis (MS) patients.

Data are presented as mean ± SEM in (B), (D), (F), (I), (K), (M), (O), (P), and (S); data in (G) and (T) are presented as change versus the control group; n = 11–14 mice per group in (B), (I) and (K), pooled from 2 out of 3 independent experiments; n = 7–9 mice per group in (D), representative of 2 independent experiments; n = 14–15 mice per group in (F), pooled from 2 out of 3 independent experiments; n = 4 mice per group in (G); n = 10 mice per group in (M), (O) and (P); n = 5 mice per group in (S) and (T); all experiments involved male mice only; two-way ANOVA with Sidak's multiple comparisons test between control and VEGF-C/D trap groups in (B), (D), (F), (I) and (K); two-tailed unpaired Student’s t-test or Wilcoxon rank sum test were used according to data normality as assessed using the Shapiro-Wilk test in (G); two-tailed unpaired Student’s t-test in (M), (O), (P), (S) and (T); simple and multiple linear regression in (U).

See also Supplementary File 1.