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Microvascular endothelial cells engulf myelin debris and promote macrophage recruitment and fibrosis after neural injury

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Author contributions

Y.R. and Y.Z. conceived the study. Y.R., Y.Z., and T.Z. designed the experiments and analyzed the data. T.Z. and Y.Z. executed most of the experiments. L.S. performed flow cytometry, guided MBP ELISA and led the sample preparation for RNA sequencing. S.R.B., H.S. and W.W. established the mouse EAE model and provided EAE spinal cord samples. Y.J. and C.L. designed the CRISPR–Cas9 knockout of Atg5. Y.L. performed spinal cord injection and some SCI. A.J.R. analyzed the RNA-sequencing data. X.W. provided the preliminary phenotype on in vivo microvessel angiogenesis after SCI. Z.C. provided some preliminary SCI tissues. Z.H. assisted in tissues collection and cryosectioning. N.Z. assisted in western blot assay and some quantifications. X.S., J.L., J.F. and W.W. analyzed some data. T.L.M. helped design some of the experiments, contributed to discussion and edited the manuscript and figures. J.L., J.F., C.L., W.W. and G.W. contributed to discussion. Y.Z., Y.R. and T.Z. wrote the manuscript.

Competing interests

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Abstract

The clearance of damaged myelin sheaths is critical to ensure functional recovery from neural injury. Here we show a previously unidentified role for microvessels and their lining endothelial cells in engulfing myelin debris in spinal cord injury (SCI) and experimental autoimmune encephalomyelitis (EAE). We demonstrate that IgG opsonization of myelin debris is required for its effective engulfment by endothelial cells and that the autophagy–lysosome pathway is crucial for degradation of engulfed myelin debris. We further show that endothelial cells exert critical functions beyond myelin clearance to promote progression of demyelination disorders by regulating macrophage infiltration, pathologic angiogenesis and fibrosis in both SCI and EAE. Unexpectedly, myelin debris engulfment induces endothelial-to-mesenchymal transition, a process that confers upon endothelial cells the ability to stimulate the endothelial-derived production of fibrotic components. Overall, our study demonstrates that the processing of myelin debris through the autophagy–lysosome pathway promotes inflammation and angiogenesis and may contribute to fibrotic scar formation.

A contusive SCI induces acute mechanical compression of myelin sheath and causes prominent demyelination, a characteristic that is also well documented in multiple sclerosis and other demyelinating diseases. The myelin loss, neuronal damage, and spinal microvasculature disruption after SCI trigger a cascade of secondary pathological processes including inflammation, glial and fibrotic scar formation that prevent tissue regeneration and functional recovery^{1–3}. Myelin debris, which is generated from the breakdown of myelin sheaths immediately after SCI, persists in the injury site and contributes to regeneration failure because myelin debris contains molecules that potently inhibit axon regeneration^{4,5} and remyelination^{6,7}. Moreover, myelin debris is actively involved in inflammatory responses during SCI progression^{8–10}. Therefore, clearance of myelin debris from the injury site is critical for axon regeneration, remyelination and resolution of inflammation.

Myelin debris is cleared mainly by ‘professional’ phagocytes such as bone marrow-derived macrophages (BMDM ϕ) and resident microglia^{10–12}. However, BMDM ϕ are not significantly recruited to the injury site until one week after SCI¹⁰, and microglia are generally absent from the lesion epicenter^{10,13}. These observations led to the hypothesis that an alternative phagocytic process performed by ‘amateur’ phagocytes present in the injury core may complement macrophages and microglia for myelin debris clearance, at least in the early stages. Indeed, a recent report shows that astrocytes act as amateur phagocytes to participate in myelin debris clearance in multiple sclerosis¹⁴. However, this cannot be the case for SCI, because astrocytes are absent from the epicenter of injured spinal cords.

Microvessels are present in the injury core as early as 3 d post injury, and their density increases up to 540% of that of normal conditions during the chronic phase of SCI^{15,16}. After acute injury, the newly formed microvessels arise via angiogenesis, or proliferation of microvascular endothelial cells. It is known that endothelial cells can act as amateur phagocytes to engulf large particles such as bacteria¹⁷, apoptotic cell bodies¹⁸ and latex particles¹⁹. Given the early presence and large number of newly formed microvessels in the injury core, we hypothesize that microvessels and the lining microvascular endothelial cells serve as amateur phagocytes for myelin debris uptake.

In the current study, we established a previously unidentified role for microvessels and lining microvascular endothelial cells in engulfing and degrading myelin debris after SCI and EAE, a commonly used animal model of multiple sclerosis. We also discovered a novel pathway for myelin debris degradation through the autophagy–lysosome system. Importantly, we demonstrated for the first time that microvascular endothelial cell uptake of myelin debris exerts critical functions beyond myelin debris clearance. Engulfment and autophagic processing of myelin debris by endothelial cells have sequential consequences in promoting chronic inflammation and pathological healing (angiogenesis and fibrotic scar formation) during the progression of demyelinating disorders.

Results

Microvessels in the demyelinating spinal cords contain myelin debris.

Microvessels in the lesion epicenter are lost during the first 2 d after SCI, whereas endothelial cells give rise to newly formed microvessels from 3 d after injury, restoring microvessel density to a normal level by one week after SCI^{15,16}. We first examined whether these newly formed microvessels could engulf myelin debris. The uninjured spinal microvessels contain little detectable myelin basic protein (MBP) (Fig. 1a,a'). By contrast, myelin debris started to closely associate with newly formed microvessels in the lesion core as early as 3 d post SCI (Supplementary Fig. 1) and became more apparent at 5 or 7 d after SCI (Fig. 1b and Supplementary Fig. 1). The *x-z* and *y-z* view of myelin debris distribution relative to microvessels revealed that myelin debris was indeed engulfed by microvessels (Fig. 1b and Supplementary Fig. 1). Myelin debris-containing microvessels were frequently observed in the injured region and were much less frequently seen in the uninjured spinal cords after SCI (Fig. 1d and Supplementary Fig. 1). Furthermore, neutral lipids, the myelin degradation products that are stained with oil red O (ORO), can be detected in microvessels at the injured core at 14 d after SCI (Fig. 1e,e').

We next investigated whether our observation extends to other demyelinating disorders such as an EAE mouse model (Supplementary Fig. 2a). The sagittal sections of EAE spinal cords demonstrated that the typical demyelinating lesions were concentrated in lumbar and thoracic cords (Supplementary Fig. 2b). We observed the myelin debris-containing microvessels in T10–T12 demyelinating segments of spinal cords after 1 week of EAE induction (Fig. 1c,c',d). These *in vivo* data from SCI and EAE models indicate that microvessels engulf myelin debris after demyelination.

In vitro BMEC-induced microvessel-like structures engulf myelin debris.

Primary mouse brain microvascular endothelial cells (BMECs) grown on Matrigel can form microvessel-like structures²⁰. After incubation with microvessel-like tubules, myelin debris was seen as scattered puncta around or within the tubules (Fig. 1f). A closer inspection of the distribution of myelin debris revealed the apparent dynamics of myelin debris entry (Fig. 1f'). Some myelin fragments appeared to be close to but were not in direct contact with the capillary surface, as indicated by a lack of colocalization with endothelial marker CD31 (Fig. 1f'). Other myelin fragments were in the process of entering, whereas others still had completely transited the luminal membrane, thus showing partial or full colocalization with CD31 (Fig. 1f'). These data confirm that myelin debris could be internalized by microvessels.

In vitro engulfment of myelin debris by BMECs.

We next investigated the kinetics and mechanisms of microvascular engulfment of myelin debris by using primary BMECs and a BMEC cell line, bEnd.3. Both primary BMECs and bEnd.3 cells engulfed myelin debris in a time-dependent manner with predominant perinuclear distribution (Fig. 2a,d). After incubation with myelin debris, the number of myelin-laden endothelial cells (myelin-ECs) and intracellular MBP were quantified by flow cytometry (Fig. 2b) and ELISA (Fig. 2c), respectively. The kinetics of myelin engulfment by bEnd.3 cells exhibited inefficient engulfment from 24–48 h, efficient engulfment from 48–72 h and saturated engulfment from 72–96 h (Fig. 2e,f). Endothelial cell uptake of myelin debris was much slower than that of BMDM ϕ , which showed rapid myelin engulfment as early as 1–3 h (Supplementary Fig. 3).

IgG opsonization is required for effective myelin debris engulfment by BMECs.

Complement-3 receptor (CR3) and Mac-2 (Glactin-3) have been proposed as receptors for myelin debris phagocytosis by macrophages²¹ (Supplementary Fig. 4a). Blockage of CR3 or Mac-2 alone by neutralizing antibodies or a combined blockage of CR3 and Mac2 did not affect myelin debris engulfment by BMECs (Fig. 2g and Supplementary Fig. 4b.). Low-density lipoprotein receptor-related protein 1 (LRP1), a proposed receptor for MBP²², was shown to mediate myelin debris phagocytosis by microglia, astrocytes and oligodendrocytes^{14,22}. However, BMEC engulfment of MBP-deficient myelin debris isolated from shiverer mice was not impaired (Supplementary Fig. 5). These data suggest that CR3, Mac-2 or LRP1 have little effect in mediating myelin debris uptake by endothelial cells.

Macrophage phagocytosis is mediated by serum-derived opsonins including antibodies and complement proteins²³. To evaluate the role of opsonins on myelin debris engulfment, we cultured BMECs with myelin debris in different concentrations of serum. BMEC engulfment of myelin debris was stronger in the presence of 5% serum than that in 1% serum (Supplementary Fig. 6) and was significantly reduced or even abolished after withdrawal of serum from interaction medium for phagocytosis (Supplementary Fig. 6), suggesting that the factor(s) in serum are required for BMEC engulfment of myelin debris. Heat inactivation of complement in serum at 56 °C failed to prevent myelin uptake by BMECs, whereas serum IgG inactivation at 70 °C^{24,25} significantly abrogated myelin debris engulfment (Fig. 2h,i),

indicating that serum IgG is required for myelin engulfment by BMECs. Supplement of IgG alone in serum-free medium or in IgG-inactivated serum rescued BMEC engulfment of myelin debris (Fig. 2i). Importantly, precoating myelin debris with IgG was sufficient for myelin engulfment by BMECs in the serum-free culture or IgG-inactivated serum culture (Fig. 2h,i). These data indicate that IgG opsonization of myelin debris is necessary and sufficient for myelin engulfment by endothelial cells.

Transcriptional profiles of endothelial cells after engulfing myelin debris.

To understand the cellular and molecular alterations in endothelial cells after myelin debris uptake, we performed RNA sequencing of endothelial cells with myelin debris engulfment (myelin-ECs) and without (naïve-ECs). Over 2,500 genes were significantly upregulated, and over 4,000 genes were downregulated in myelin-ECs compared with naïve-ECs (Supplementary RNA sequencing Dataset, Fig. 3a). The differentially expressed genes in myelin-ECs were enriched in a variety of processes or signaling pathways, mainly including metabolism, extracellular matrix formation, vesicle transport, inflammation and cell junctions, among others (Supplementary Table 1 and Fig. 3b,c).

Noteworthy, among the top 50 upregulated genes in myelin-ECs were collagen genes, including *Col1a2*, *Col5a2*, *Col16a1*, *Col6a2*, all of which were remarkably upregulated (Fig. 3b), confirmed by qPCR (Fig. 3d). The inflammatory genes including interleukin (IL)-related genes (*Il1rl1*, *Il-4*, *Il-5*, *Il13ra1*) and chemokine genes (*Ccl2* (also known *Mcp-1*) and *Cxcl1*) were also upregulated in myelin-ECs (Fig. 3b). qPCR analysis confirmed the increased gene expression of *Il-4*, *Il-6*, *Mcp-1* and *iNOS* in myelin-ECs (Fig. 3e). Myelin debris uptake also upregulated vesicle-encoding genes for lysosomes, autophagosomes and endosomes (Fig. 3b). The downregulated genes in myelin-ECs are involved in the Notch signaling pathway and cell adhesion and junction (Fig. 3c), which is related to endothelial angiogenesis and permeability, respectively.

Engulfed myelin debris is delivered to lysosomal degradation system through autophagy pathway.

We next examined subcellular localization of engulfed myelin debris. Myelin debris was predominantly delivered to lysosomes, as revealed by colocalization between the majority of myelin particles and puncta positive for Lysotracker red (Fig. 4a,a'). Myelin debris uptake increased the size of the lysosomes, especially those containing myelin debris (Fig. 4a',b). Lysosomes in naïve-ECs were $0.60 \pm 0.02 \mu\text{m}$ in diameter (Fig. 4a',b). However, lysosomes that contained myelin debris were $1.35 \pm 0.03 \mu\text{m}$ whereas lysosomes containing no myelin debris in myelin-ECs were maintained around $0.64 \pm 0.01 \mu\text{m}$ (Fig. 4a',b).

Cargo can be delivered through endocytosis or autophagy pathways to lysosomes for degradation²⁶. We first examined whether myelin debris colocalizes with endosomes in BMECs. There was almost no or little colocalization between myelin debris and the early (Rab5) and late (Rab7) endosomes (Fig. 4c,d), thus suggesting that endosomal machinery is not the primary route for myelin delivery to lysosomes.

RNA sequencing data revealed upregulation of autophagy genes, including *Gabarapl2*, *Gabarap*, *Atg12*, *LC3b*, *Atg5* and *Atg3* in myelin-ECs (Fig. 3b). We next explored the

possible role of autophagy in delivering myelin debris to lysosomes. Myelin-ECs increased autophagosome formation (LC3b-puncta) considerably, whereas fed BMECs showed few LC3b-puncta (negative control), and starvation induced robust formation of LC3b-puncta (positive control) (Fig. 4e). A closer look at the relative localization between myelin debris and LC3-puncta revealed that most myelin debris was in contact with autophagosomes (Fig. 4c,d). Myelin debris also showed significant colocalization with puncta positive for another autophagy marker, GABARAP (Fig. 4c,d). The western blot analysis demonstrated that myelin debris uptake induced LC3-I conversion to LC3-II (Fig. 4f and Supplementary Fig. 13), an indicator of autophagosome formation. Moreover, myelin debris uptake caused autophagy substrate p62 degradation (Fig. 4f), indicating autophagy induction by myelin debris.

We next examined whether inhibiting the autophagy–lysosome pathway could block myelin degradation in *Atg5* knockout (*atg5*^{−/−}) BMECs generated using CRISPR–Cas9 (Fig. 4g, Supplementary Fig. 7a,b and Supplementary Fig. 13). The *atg5*^{−/−} BMECs maintained normal cell viability (Supplementary Fig. 7d). The *atg5*^{−/−} BMECs failed to generate LC3⁺ puncta (Fig. 4h) and induced LC3 conversion (Fig. 4i, Supplementary Fig. 13), as well as accumulated p62 and ubiquitin (Fig. 4i, Supplementary Fig. 7c), verifying the knockout is functional. *Atg5*^{−/−} BMECs failed to degrade the engulfed myelin debris into neutral lipids (Fig. 4j,k). Consistently, either blocking autophagy using 3-MA, an inhibitor of autophagosome formation, or inhibition of lysosomal activity with chloroquine, significantly inhibited myelin degradation into neutral lipids in myelin-ECs without causing apparent cell toxicity (Fig. 4j,k, Supplementary Fig. 7d). Conversely, additional supply of autophagosomes to myelin-ECs accelerated myelin degradation by rapamycin or starvation treatment (Fig. 4j,k). These genetic or pharmacological data indicate that autophagy–lysosome pathway is required for engulfed myelin debris degradation.

Microvessels are enlarged in SCI and EAE models.

Although the microvessel density is remarkably increased during the subacute phase of SCI^{15,16}, little attention has been paid to the change in the morphology and structure of the newly formed microvessels. Compared with the microvessels with a mean diameter of $8.17 \pm 0.41 \mu\text{m}$ in normal spinal cords, the microvessels in the injured core increased the mean diameter to $16.66 \pm 0.51 \mu\text{m}$ after 1 week post SCI (Fig. 5a,b and Supplementary Fig. 8a). These dilated microvessels were not only seen in the injured core at 1, 4, 6, 8 and 10 weeks after SCI, but also seen in the marginal regions (Fig. 5a,b). The dilated microvessels in the injury region were proliferative, as indicated by Ki-67, a marker for cell proliferation (Fig. 5c,c'). Interestingly, microvessels in demyelinated regions were also enlarged (Fig. 5d,d') and proliferative in the spinal cords from EAE mice (Fig. 5e). These data indicate that enlarged microvessels are common in demyelinating diseases.

Engulfment and autophagic processing of myelin debris promote endothelial cell proliferation and angiogenesis.

We next sought to identify whether lesion-related factors stimulate microvascular growth. Myelin debris uptake significantly increased the Ki-67⁺ proliferative endothelial cells (Fig. 5f). Cell populations were increased after exposure to myelin debris in a time-dependent

manner (Fig. 5g). The proliferative capacity of myelin-ECs was further confirmed using an *in vivo* Matrigel angiogenesis assay. Subcutaneous injection of Matrigel plugs containing myelin-ECs stimulated extensive angiogenesis (Fig. 5h). Similarly, myelin-ECs injected into normal spinal cord remained highly proliferative and appeared integrated within microvasculature (Supplementary Fig. 8b). It is noteworthy that both injection of myelin debris alone and injection of endothelial cells (endothelial cells were exposed to but not able to engulf myelin debris in the presence of IgG-inactivated serum) failed to induce angiogenesis (Supplementary Fig. 8b). Moreover, *in vitro* myelin-induced endothelial cell proliferation was abrogated after *Atg5* knockout (Fig. 5i), indicating that autophagic degradation of myelin debris is required for endothelial cell proliferation. These data indicate that myelin debris gains proangiogenic potential only after being engulfed and intracellularly processed in endothelial cells. The proangiogenic potential of myelin-ECs could likely be attributed to the increased expression of vascular endothelial growth factor (VEGF) (Fig. 5j), as VEGF neutralization blocked myelin-induced endothelial cell proliferation (Fig. 5k).

Endothelial cell engulfment of necrotic cells inhibits cell proliferation.

We wondered whether the necrotic cells generated after SCI could also affect endothelial cell proliferation and angiogenesis. BMECs were able to engulf the necrotic neuronal cells (Supplementary Fig. 9a,b), but less efficiently compared with BMDM ϕ (Supplementary Fig. 9c). Unlike myelin uptake, necrotic cell engulfment significantly inhibited BMEC growth (Supplementary Fig. 9d). We tested this specificity in two additional assays. Zymosan, or dead yeast, was efficiently engulfed by BMDM ϕ (Supplementary Fig. 9g) but could not be engulfed by BMECs (Supplementary Fig. 9h) and had no effect on endothelial cell proliferation (not shown). We next tested whether the reactive astrocytes, which form glia, scar around SCI lesions and are spatially distant from microvessels in the injured core. We mimicked this indirect interaction in a transwell assay (Supplementary Fig. 10a,b) and found that resting astrocytes and LPS-induced reactive astrocytes had no significant effects on BMEC proliferation (Supplementary Fig. 10c).

Myelin debris uptake by endothelial cells stimulates inflammatory responses.

We next sought to examine whether myelin debris-primed endothelial cells promote inflammation by inducing leukocyte infiltration. We previously demonstrated that the bone marrow-derived cells that infiltrated the injured spinal cord are mainly BMDM ϕ using GFP⁺ bone marrow chimeric mice¹⁰. Normal spinal cords had little or no GFP⁺ BMDM ϕ infiltration. However, GFP⁺ BMDM ϕ infiltrated in the injured core and closely associated with newly formed microvessels after 3 d post SCI (Fig. 6a and Supplementary Fig. 11a). The number of microvessel-associated BMDM ϕ increased from 3 d to 1 or 2 weeks post SCI (Fig. 6a and Supplementary Fig. 11a), thus correlating with formation of enlarged microvessels in the injury core. Similarly in 1-week EAE mice spinal cord, infiltrated Iba-1⁺ macrophages closely associated with enlarged microvessels in demyelinating regions (Fig. 6b and Supplementary Fig. 11b). This correlation suggests that macrophage recruitment is dependent on the newly formed microvessels, which may serve as a portal to facilitate BMDM ϕ entry. Supporting this, a closer look at the infiltrated BMDM ϕ showed that some BMDM ϕ were bordering the outer surface of microvessels or were just entering the spinal

cord (Fig. 6a'), indicating that they were in the process of or had completed transmigration across microvascular endothelium toward the spinal cord parenchyma.

We next determined whether myelin debris uptake activates endothelial cells, a critical step for leukocyte infiltration. There were a greater number of BMDM ϕ adhering to the myelin-EC monolayer than that to the naïve-ECs (Fig. 6c,c'), probably due to the increased expression of vascular cell adhesion molecule-1 (VCAM-1) in myelin-ECs (Fig. 6d and Supplementary Fig. 13). Endothelial cells did not increase BMDM ϕ adhesion when exposed to myelin debris without IgG opsonization (Fig. 6c' and Supplementary Fig. 11c), where myelin debris was exposed to ECs without being engulfed. Endothelial cells with myelin uptake but no intracellular degradation (*atg5*^{-/-} endothelial cell uptake myelin debris) did not increase BMDM ϕ adhesion either (Fig. 6c' and Supplementary Fig. 11c), indicating that only intracellularly processed myelin debris is able to activate endothelial cells and induce BMDM ϕ adhesion. Aside from macrophage adhesion, conditioned media from myelin-ECs, but not from naïve-ECs, remarkably stimulated BMDM ϕ chemotaxis (Fig. 6e), suggesting that endothelial-derived factors from myelin-ECs stimulate BMDM ϕ migration. We found that myelin debris significantly increased endothelial secretion of MCP-1 (Fig. 6f), the major chemokine for macrophage recruitment²⁷. Myelin-ECs also upregulated other pro-inflammatory mediators (Fig. 3b,e), which may together contribute to BMDM ϕ infiltration. Furthermore, myelin-ECs activated BMDM ϕ , as evidenced by upregulated IL-6 expression in BMDM ϕ after being exposed to myelin-EC-conditioned media (Fig. 6g). We then examined the inflammatory potential of myelin-ECs by injection of naïve-ECs or myelin-ECs into normal spinal cords. Myelin-EC injection increased the number of Iba-1⁺ macrophages and microglia and activated astrocytes compared with injection of naïve-ECs (Fig. 6h and Supplementary Fig. 11e,f). It is noteworthy that injection of myelin alone or endothelial cells (endothelial cells were exposed to but not able to engulf myelin debris in the presence of IgG-inactivated serum) failed to induce inflammation in vivo (Supplementary Fig. 11d-f), confirming that myelin debris uptake by endothelial cells, but not simple exposure, promotes pro-inflammatory responses. Furthermore, we showed that engulfment of necrotic neuronal cells had no significant change in *Mcp-1* gene expression (Supplementary Fig. 9e), indicating the specificity of endothelial cell engulfment of myelin debris in promoting inflammation.

Enlarged microvessels and endothelial cells contribute to fibrotic components production.

A fibrotic scar forms and occupies the injury core, which is typically characterized by extensive deposition of collagen²⁸ and fibronectin²⁹. However, little is known about the cellular origin of such fibrotic scar in the injury core. Given the highly upregulated collagen genes in myelin-ECs (Fig. 3b,d), we therefore examined the spatiotemporal relationship between microvascular endothelial cells and collagen and fibronectin in the injured spinal cords. A low level of collagen 1 (Col1) expression in the normal cord was detected, with a close association with microvessels (Fig. 7a). However, at 6 weeks after SCI, dense Col1 was present at the injury core, and its expression pattern intimately resembled that of enlarged microvessels (Fig. 7b). There was almost no expression of fibronectin throughout the normal spinal cords (Fig. 7d). Some but not all microvessels were closely associated with fibronectin expression at 6 weeks after SCI (Fig. 7e). Similarly, a dense Col1 or

fibronectin colocalized well with enlarged microvessels in the spinal cords from EAE mice (Fig. 7c,f). Our in vivo data from two demyelinating models support that microvascular endothelial cells could be a novel source of fibrotic scar formation through endothelial production of collagen and fibronectin.

RNA sequencing identified the top upregulated genes implicated in fibrosis, including collagen genes *Col1a2*, *Col5a2*, *Col16a1*, and *Col16a2* in myelin-ECs (Fig. 3b), suggesting that myelin debris has the potential to stimulate endothelial cells to produce fibrotic components. BMECs treated with myelin debris for 1 d did not significantly increase expression of Col1 (data not shown) or fibronectin (Fig. 7i and Supplementary Fig. 13); however, prolonged treatment with myelin debris significantly increased expression of Col1 (Fig. 7g,g') and fibronectin (Fig. 7i) to a level similar to that of transforming growth factor β 1 (TGF- β 1) treatment, a strong inducer of collagen or fibronectin production³⁰. Myelin debris also increased Col1 expression in microvessel-like structures (Fig. 7h), supporting the notion that myelin debris stimulates microvascular production of pro-fibrotic proteins in the lesion core.

Engulfment and autophagic processing of myelin debris by endothelial cells induce endothelial-to-mesenchymal transition.

Interestingly, treatment with myelin debris for 10 d reduced CD31 expression in some BMECs (Fig. 7g), a phenotype resembling endothelial-to-mesenchymal transition (endoMT), which has been implicated in tissue fibrosis in various diseases³¹. Control BMECs exhibited the characteristic polygonal cobblestone-like morphology (Fig. 8a,c), whereas after treatment with TGF- β 1, a strong inducer of endoMT³¹, and myelin debris for 10 d, BMECs elongated and exhibited the same spindle-like morphology as fibroblasts (Fig. 8a,c), a morphological change indicating the induction of endoMT. Additionally, exposure to TGF- β 1 or myelin debris, but not to necrotic cells, markedly downregulated CD31 expression and strongly induced the expression of endoMT marker α -smooth muscle actin (α -SMA) in BMECs (Fig. 8b,d–f and Supplementary Fig. 9f), indicating the specificity of myelin uptake by endothelial cells in endoMT induction. Moreover, engulfment of myelin debris upregulated TGF- β 1 expression (Fig. 8g), and blockade of TGF signaling by a pan-TGF- β neutralizing antibody abrogated myelin-induced endoMT phenotypes (Fig. 8a–d). Autophagic processing of myelin debris was crucial for myelin-induced endoMT because *atg5*^{−/−} BMECs failed to show morphological change (Fig. 8a,c) and α -SMA expression (Fig. 8b,d). Noteworthy, at 6 weeks after SCI and 15 d of EAE, microvessels colocalized with CD31 and α -SMA, indicating in vivo endoMT after demyelination (Fig. 8h). Our data showed that myelin debris stimulates endothelial-derived fibrotic components, probably via endoMT.

Discussion

Although BMDM ϕ and microglia are the major phagocytes to clear myelin debris generated after demyelination, our data demonstrated that microvascular endothelial cells act as amateur phagocytes to engulf myelin debris. We revealed that myelin debris uptake and autophagic processing by microvascular endothelial cells have more important functions.

Endothelial cell uptake and processing of myelin debris cause a series of sequential events associated with disease progression, including inflammation, angiogenesis and fibrotic scar formation.

Most of our knowledge of myelin debris phagocytosis comes from studies on macrophages and microglia. Receptors such as CR3, Mac-2 and LRP-1 are involved in myelin debris phagocytosis by macrophages and microglia²¹. Our study showed that endothelial cells do not employ these receptors for myelin debris uptake. The ‘naked’ myelin debris is not recognized by endothelial cells, and only IgG-opsonized myelin debris can be engulfed effectively, suggesting that IgG receptors (FcγRs) are involved in myelin debris engulfment by endothelial cells. A compromised blood–brain barrier leads to leakage of IgG in the injured area³², which may be the source of IgG for opsonization. Given the fact that brain endothelial cells and other antigen-presenting cells are able to engulf myelin debris and present myelin antigens to lymphocytes³³, the specific antibodies may further opsonize myelin debris and facilitate its engulfment. Endogenous antibodies have been shown to promote the rapid clearance of myelin debris in mouse³⁴, but it is unknown which cell type(s) benefit from the opsonization by antibody for myelin debris uptake. We speculate that endothelial cells are the major cell type that relies on antibody opsonization of myelin debris for myelin debris clearance.

Autophagy is a fundamental degradative pathway for degradation of intracellular proteins and organelles. Autophagy has recently emerged as an alternative mechanism for myelin debris clearance in Schwann cells^{35,36}. Using autophagy-deficient endothelial cells, we show that autophagy is required for myelin debris degradation in endothelial cells. Furthermore, autophagic processing of myelin debris is crucial for pro-angiogenic, pro-inflammatory, and pro-fibrotic responses. However, if endothelial cells either contact myelin debris but do not internalize it or internalize myelin debris without autophagic processing, those responses are not elicited, thus indicating that myelin debris causes consequences only after being engulfed and intracellularly processed.

The major vascular change in the injury area is angiogenesis during chronic stages of SCI. The newly formed microvessels in both SCI and EAE models are structurally abnormal, appearing dilated and more disorganized. The mechanisms and biological outcomes for these vascular abnormalities are poorly understood after demyelination. We demonstrated that myelin debris is one critical lesion-related factor that causes excessive endothelial cell proliferation, which may contribute to microvessel dilation at injury sites. Interestingly, the dilated microvessels in the injured spinal cords recapitulated the microvessels in mice lacking pericytes³⁷, a cellular constituent in the neurovascular unit that has been recently reported to constrict microvessels³⁸. Therefore, an alternative explanation for the microvessel dilation could be that these newly formed microvessels have defects in pericyte maturation or/and coverage, which thus fail to constrict microvessels and lead to microvessel dilation.

One of the most important features of neuroinflammation is the leukocyte recruitment, which requires the endothelial cell activation through an increased expression of VCAM-1 and cytokines and chemokines³⁹. Myelin debris engulfment activates endothelial cells by

increasing expression of VCAM-1 and a variety of cytokines and chemokines that could facilitate BMDM ϕ recruitment to the injury site. Myelin debris might also promote BMDM ϕ influx to the injury site by increasing microvascular permeability to leukocytes, as indicated by the downregulation of genes related to cell junctions in myelin-ECs.

The fibrotic scars in the central region of injury sites, characterized by the excessive accumulation of collagen and fibronectin, have been known to inhibit axon regeneration⁴⁰. Fibroblasts, which are prominent in the injured epicenter, contribute to fibrotic scar formation by stimulating the production of collagen²⁸ and fibronectin²⁹. However, little is known about the cellular origin of fibroblasts in contusive injured spinal cords^{28,29,41}. Our study demonstrated that enlarged microvessels contribute to the significant deposition of fibrotic components in SCI and EAE models. Soderblom *et al* reported contribution of perivascular fibroblasts from larger-diameter microvessels (in our study, we referred to them as enlarged or dilated microvessels) to Coll1 α 1 production and fibrotic scar formation using a Coll1 α 1-GFP transgene, which is consistent with our results²⁸. The exact cellular identity of perivascular fibroblasts is unclear, given that different cell types can share the same cell markers, and some cell types can have further subtypes.

The activated fibroblasts, or myofibroblasts, may arise from other sources, including resident fibroblasts, perivascular pericytes, bone-marrow-derived precursors and others⁴². Endothelial cells have greater plasticity and can acquire fibroblast-like properties by undergoing endoMT³¹. Our study demonstrated that endothelial cells could become fibroblast-like cells after myelin uptake via endoMT, suggesting that microvascular endothelial cells are an additional source of fibroblasts or fibroblasts-like cells for fibrotic scar formation at the SCI lesion core. Interestingly, it takes a few days for myelin debris to significantly increase expression of fibronectin, collagen, and α -SMA in microvascular endothelial cells, coinciding with the delayed accumulation of perivascular fibroblasts at the injury core⁴³. We further determined that myelin debris induces endoMT via TGF- β 1. TGF signaling has been known as a master regulator of endoMT³¹ and participates in the formation of fibrotic scars in the injury site^{44,45}. The TGF- β signaling is activated in several cell types within SCI lesions, including macrophages, astrocytes and endothelial cells^{40,46}. Thus, we propose that TGF signaling-mediated endoMT in endothelial cells may underlie the effects of TGF signaling on fibrotic scar formation in SCI lesions.

In conclusion, we reveal that microvessels and lining microvascular endothelial cells act as amateur phagocytes to engulf myelin debris generated by CNS disorders associated with prominent demyelination. Mechanistically, we determined that IgG opsonization of myelin debris is required for efficient uptake by microvascular endothelial cells. The engulfed myelin debris is then delivered through autophagy-lysosome pathway for intracellular degradation. Functionally, engulfment and autophagy-dependent processing of myelin debris by microvascular endothelial cells contribute to three critical processes that are closely associated with CNS demyelinating disorders: robust angiogenesis that results in excessive and abnormal microvessels, chronic inflammation, and endo-thelial-mediated fibrosis (Supplementary Fig. 12). Therefore, it may be possible to reverse the effects of myelin-ECs by targeting these particular processes (for example, myelin debris uptake, autophagy and endoMT).

Methods

Reagents.

Chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO), and cell culture media was purchased from Invitrogen (Carlsbad, CA), unless otherwise indicated. Carboxyfluorescein succinimidyl ester (CFSE; #C1157) was purchased from Life Technologies (Carlsbad, CA). 3-Methuladenine (3-MA; BML-AP502) was purchased from Enzo Life Sciences (Farmingdale, NY). and rapamycin (#553210) was purchased from EMD Millipore (Burlington, MA). Recombinant mouse TGF- β 1 (#5231) was from Cell Signaling Technology (Danvers, MA). Mouse MCP-1 ELISA kit (#432701) was from Bio Legend (San Diego, CA). Matrigel matrix growth factor reduced (#354230) was from BD Biosciences (San Jose, CA). LysoTracker red DND-99 (L7528; 1:5,000 for staining) was purchased from Invitrogen.

Antibodies.

Anti-CD31 (#550274; 1:100 for immunofluorescence (IF)) was from BD Biosciences (Franklin Lakes, NJ). Anti-MBP (ab40390; 1:200 for IF; 1:1,000 for ELISA), anti-Ki-67 (ab15580; 1:200 for IF), anti-CD11b (ab133357; 1:100 for IF), anti-Von Willebrand Factor (vWF; ab11713; 1:100 for IF), anti- α -SMA (ab124964; 1:400 for IF), anti-fibronectin (ab23750; 1:200 for IF; 1:1,000 for WB), anti-collagen I (ab34710; 1:200 for IF), anti-GFAP (#ab53554, 1:400) and GAPDH (ab181602; 1:3,000 for western blot (WB)) were purchased from Abcam (Cambridge, MA). The antibodies against LC3 (#4108; 1:100 for IF; WB 1:1,000), Atg5 (D5F5U, #12994, 1:1,000 for WB), Rab5 (#3547; 1:50 for IF), Rab7 (#9367; 1:100 for IF) and GABARAP (#13733; 1:200 for IF) were purchased from Cell Signaling Technology (Danvers, MA). Another anti-MBP (MAB386; 1:1,000 for ELISA) was purchased from Millipore (Billerica, MA), anti-VCAM-1 (sc-8304; 1:250 for WB) was purchased from Santa Cruz Biotechnology (Dallas, TX) and anti-Lamp1 (1D4B; 1:25 for IF) was from Developmental Studies Hybridoma Bank. Anti-tubulin (DM1A; 1:5,000 for WB) was from Sigma-Aldrich (St. Louis, MO). Anti-p62 (PM045; 1:1,000 for WB) was from MBL (Woburn, MA). Anti-ubiquitin (FK2, #BML-PW8810-0500; 1:200 for IF) was from ENZO Life Sciences. Anti-Iba-1 (#019-19741, 1:200 for IF) was from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). F4/80 and Mac-2 antibodies were produced via hybridoma cell lines (HB-198 for F4/80; TIB-166 for Mac-2) from American Tissue Culture Collection (ATCC, Manassas, VA). VEGF neutralizing antibody (#AF-493-NA, 20 μ g/ml for neutralization) and pan-TGF- β neutralizing antibody (#MAB1835R-100, 20 μ g/ml for neutralization) were from R&D Systems (Minneapolis, MN). Alexa Fluor 488-, 555- and 647-conjugated secondary antibodies (1:600 for IF) and HRP-conjugated secondary antibody (1:3,000 for ELISA) were purchased from Invitrogen. IRDye-800CW- and IRDye-680LT-conjugated secondary antibodies (1:20,000 for WB) were from LI-COR Bioscience.

Mice.

C57BL/6J, C57BL/6-Tg (ACTB-EGFP)10sb/J and C3Fe.SWV-MBP^{shi}/J mice were purchased from Jackson Laboratory (Bar Harbor, ME) and maintained in a pathogen-free animal facility in Florida State University. All animal protocols were approved by the Animal Care and Use Committee (ACUC) of Florida State University; the EAE induction

protocol was approved by the Committee for Use of Live Animals in Teaching and Research at The University of Hong Kong. No statistical methods were used to predetermine sample sizes, and our sample sizes were chosen according to standard practices in the field. Littermates were ear tagged and randomly assigned to different experimental groups. All results from animal work were evaluated independently from two blinded experienced researchers. Quantification of microvessel diameter was performed independently by two blinded experienced researchers.

Spinal cord injury in mice.

Thoracic spinal cord contusion injuries were performed on 8- to 10-week old C57BL/6J female mice. To expose the spinal cord, a laminectomy was performed on the T10 vertebrae. The contusion injury was induced using the NYU impactor with a 5 g rod dropped 6.25 mm from the cord surface⁴⁷. Mice in the sham group were subjected to a laminectomy without a contusion.

Active EAE induction in mice.

EAE induction was performed as described previously with minor modifications^{48,49}. All of the animal experiments were approved by the Committee for Use of Live Animals in Teaching and Research at The University of Hong Kong. 7- to 8-week-old female C57BL/6J mice were used for EAE induction. The animals were housed in the Laboratory Animal Unit on a 12 h day/night cycle, with food and water ad libitum, and were allowed to acclimatize for 1 week before disease induction.

Female mice were subcutaneously immunized with 200 µg MOG35–55 peptide (Genscript, Piscataway, NJ) in complete Freund's adjuvant (3 mg/ml). Freshly prepared pertussis toxin (PHZ1174, ThermoFisher, 250 ng) in sterile PBS was injected intraperitoneally on day 0 and 48 h later.

EAE symptoms were scored daily as follows: 0, no clinical signs; 0.5, partially limp tail; 1, paralyzed tail; 1.5, hind limb paresis or loss in coordinated movement; 2, loss in coordinated movement and hind limb paresis; 3, one hind limb paralyzed; 4, both hind limbs paralyzed; 5, hind limbs paralyzed, weakness in forelimbs; 6, moribund.

Generation of GFP⁺ bone marrow chimeras.

GFP⁺ bone marrow chimeric mice were generated according to previous publication¹⁰. Briefly, female C57BL/6 mice 8–10 weeks of age were exposed to irradiation, 10 Gy with X-ray, and intravenously injected with 5×10^6 bone marrow cells freshly collected from transgenic mice (C57BL/6-Tg(ACTB-EGFP)10sb/J) constitutively expressing GFP. Efficient reconstitution was confirmed by postmortem examination of circulating blood for GFP⁺ cells. On average, 80% transplant engraftment efficiency was achieved.

Cell cultures.

Source and culture information on primary mouse brain microvascular endothelial cells, BMEC cell line bEnd.3, mouse neuroblastoma cell line Neuro-2a and BMDMφ are provided in Supplementary Methods.

Generation of Atg5-knockout endothelial cell line using CRISPR–Cas9.

A single guide RNA sequence, atgaaggcacacccctgaaa, was selected to target the third exon of the mouse Atg5 gene. The expression of guide RNA and scaffold RNA is driven under the U6 promoter. The U6 promoter and guide and scaffold cassette were incorporated into a Cas9-expressing backbone vector tagged with EGFP. The sequence of the U6 promoter, guide RNA and scaffold RNA were confirmed via the sequencing, then transfected into mouse endothelial cells line bEnd.3 using FuGENE® 6 (#E2693, Promega), then sorted by the FACS sorting process. The method for CRISPR–Cas9 has not been published, and a full characterization of this method will be published elsewhere. 48 h later, cells were trypsinized into single cells and sorted according to the GFP⁺ signal into 96-well plate. After colony expansion, the protein expression of Atg5 was analyzed using western blotting. The genomic DNA was extracted from clones that completely lost Atg5 protein expression, then the targeted region was PCR amplified with the primer set flanking the targeted region. PCR amplicons were purified with the PCR purification kit (#28104, QIAGEN), and a T7E1 (#E3321, NEB) assay was carried out to confirm the mismatch occurred at the specific site.

Preparation and fluorescent labeling of myelin debris.

Myelin debris was isolated as described previously⁹. Detailed information is provided in Supplementary Methods.

Myelin debris uptake assay.

Carboxyfluorescein succinimidyl ester (CFSE)-labeled myelin debris was added to the BMECs cultures for indicated time periods at a final concentration of 1 mg/ml. Non-ingested myelin debris was washed away from the cell surface with EDTA for 30 s and citric acid for 1 min. Myelin debris uptake was analyzed by standard assays including confocal fluorescent imaging, flow cytometry and ELISA detection of intracellular MBP, as described below.

Detailed information on the role of CR3 or Mac-2, serum concentration and IgG or complement opsonization in endothelial cell uptake of myelin debris is provided in Supplementary Methods.

Flow cytometry analysis of myelin debris uptake.

BMECs were treated with or without CFSE-labeled myelin debris for 72 h and washed to remove non-ingested myelin debris. BMECs were collected and resuspended in PBS, then subjected to immediate detection with a BD FACS Canto flow cytometer (Becton Dickinson).

Enzyme-linked immunosorbent assay detection of engulfed myelin debris.

To detect the engulfed myelin debris in endothelial cells, we performed MBP-specific sandwich ELISA as previously described^{13,50}, with rabbit MBP antibody as the capturing antibody and rat MBP antibody as the detecting antibody. Detailed information is provided in Supplementary Methods.

Myelin debris engulfment by microvessel-like structures.

Primary BMECs were seeded on the polymerized Matrigel and cultured at 37 °C for 24 h to form the tubular structures and then incubated with CFSE-myelin debris. After removal of non-ingested myelin debris, cells on Matrigel-coated coverslips were fixed with 2% paraformaldehyde (PFA), followed by regular immunostaining. Images were acquired with Nikon A1 laser scanning confocal microscope (Nikon, Japan) and the slice view of the tubular structures from both *x-y* axis and *x-z* axis were collected from the Nikon Elements analysis software.

Histology and immunofluorescent staining.

See Supplementary Methods for details.

Oil Red O staining.

ORO staining was performed to detect intracellular neutral lipid accumulation in injured spinal cords and cultured BMECs. Spinal frozen sections or fixed cells were dehydrated in 100% propylene glycol for 5 min, then stained with 0.5% ORO solution at 60 °C for 8 min. The samples were then processed with 85% propylene glycol for 5 min, then rinsed with distilled water three times. Stained samples were imaged with a confocal laser scanning microscope.

Histology quantification.

Quantifications of microvessel size were performed by unbiased researchers. To quantify the size of microvessels in three consecutive regions of SCI (injured, marginal and uninjured), we first classified the three regions with GFAP staining as a major reference according to a previous publication with modification⁵¹. Using Nikon NIS-Elements software, the total area of the spinal cord and the area of the GFAP⁺ regions were outlined and measured at 200 μ m intervals over a 2 mm distance, centered on the lesion core. The injured regions were defined as the regions spanning with a radius of around 300 μ m, which were negative for GFAP but densely positive for nuclear staining (Hoechst staining). The marginal regions, within the GFAP⁺ glia scar, were considered as 300–600 μ m away from the epicenter, and the uninjured regions were considered as regions >600 μ m away from the epicenter. We usually analyzed the normal regions that were more than 1,000 μ m away from epicenter. For microvessel size quantification in mouse EAE spinal cords, we measured microvessel diameter in both non-demyelinated and demyelinated regions in T10 segment. At least 20 microvessels with clear CD31 signals on each region were included for diameter analysis using image J Pro Plus 6.0 (Media Cybernetics, Rockville, MD).

To quantify the microvessel uptake of MBP⁺ myelin debris in mouse SCI samples, we focused on uninjured and injured regions as classified above. The uninjured region represents the region without myelin debris, and the injured region represents the region that accumulates myelin debris. The microvessel uptake of MBP⁺ myelin debris in mouse EAE samples was quantified in T10 regions at early time points (pre-onset stage). *x-y*, *x-z* and *y-z* views were included to carefully assess the presence of myelin puncta within microvessels. Microvessels containing at least one fluorescently clear MBP⁺ puncta were considered as myelin-containing microvessels.

To quantify the Ki-67-positive microvessels in SCI samples, the microvessels positive for both Ki-67 and CD31 staining were counted in the injured regions of 1 week post SCI or normal spinal cords.

For quantification of GFP⁺ BMDC infiltration across microvessels at different time points of SCI, we stained spinal cord tissues with CD31 in GFP⁺ bone marrow chimeric mice after SCI and counted the number of GFP⁺ BMDCs that are closely associated with microvessels in one whole field with an area of 0.044 mm².

For quantification of Iba-1⁺ cells in EAE microvessels, we stained Iba-1 and CD31 in T10 segment of 7 d and 15 d post-EAE spinal cords and counted the number of Iba-1⁺ cells showing close association with normal-sized or enlarged microvessels.

Autophagy assays, measurements and colocalization analysis.

Details on autophagy markers LC3 and GABARAP staining and analysis in Supplementary Methods.

Lysotracker red staining and analysis.

Details in Supplementary Methods.

Starvation and drug treatments for autophagy assays.

Details in Supplementary Methods.

Propidium iodide staining assay for cell death analysis.

The fixed BMECs were stained with propidium iodide and Hoechst, followed by imaging and quantification of propidium iodide-positive nucleus using Nikon Element software. More information in Supplementary Methods.

Image acquisition.

Samples from spinal cords and cell culture were imaged with a Nikon Ti-E microscope (Nikon Instruments, Melville, NY) using 10× objective for large image acquisition with 25% overlapping. Regions of interest were imaged with Nikon A1 laser scanning confocal microscope using a 20× objective or a 60×/1.49NA oil immersion objective. All confocal images were acquired with a spacing of 0.25 μm or 0.5 μm between z sections in Nikon NIS-Elements software and are maximum intensity projections of z stacks. In some images, volume view of *xyz* axes with or without 3D rendering and slice view of *x-y*, *x-z* or *y-z* axes were applied. Gamma correction was applied in some images.

RNA sequencing and data analysis.

Mouse brain microvascular endothelial cells (bEnd.3) were plated at equal density in cell culture dishes and allowed to rest overnight prior to addition of 1 mg/ml myelin debris, prepared as described previously. Total RNA was isolated from each of two biological replicates for control and myelin-debris-treated cells for 72 h using the TRIzol® Plus RNA Purification Kit (Thermo Fisher). Selection of mRNA from total RNA was accomplished using the NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB #E7490). From the

total mRNA obtained, cDNA was generated using the high-fidelity ProtoScript II Reverse Transcriptase (NEB) with a random primer mix to generate fragments. The double-stranded cDNA was purified using 1.8× Agencourt AMPure XP Beads prior to end preparation for adaptor ligation. The ligation reaction was performed using AMPure XP Beads and enriched via PCR followed by a final purification using the Agencourt AMPure XP Beads. Quality of the resulting library was examined using Agilent High Sensitivity DNA Bioanalyzer Chips (Agilent Technologies 5067–4626) and quantified by KAPA Library Quantification Kits for Illumina sequencing platforms (KAPA Biosystems KK4824). Single-end sequencing was performed on the Illumina HiSeq 2000 DNA Sequencer in the Florida State University Department of Biomedical Sciences Translational Sciences Facility.

For mRNA-seq data analysis, the resulting sequences were trimmed of their Illumina indexing adaptors using Trimmomatic⁵². All reads between 50 and 100 bases were included in further analyses. Any reads with greater than 2 Ns were considered to be low quality and thus discarded. Unique reads were aligned to the *Mus musculus* genome using TopHat2. A total of four mismatches between the reference genome and sample was allowed during alignment to account for strain differences between the endothelial cell line and the C57BL/6 reference genome. The TopHat2 mapped reads were further processed (filtered, sorted and indexed) with Samtools such that reads mapped to a single gene were used for further analysis^{53,54}. The uniquely mapped reads were then used to generate counts for each annotated gene using HTSeq (from Bioconductor version 3.0.2)⁵⁵. Finally, differential expression analysis of count tables for control versus myelin debris treated comparisons at each time point was performed in DESeq2 (1.8.1, Benjamini–Hochberg false discovery rate correction)^{56–58}.

Quantitative RT-PCR.

Total RNA from cells was extracted using TRIzol. cDNA was reverse transcribed from 1 µg of RNA using qScript Flex cDNA Synthesis Kit (#95047; Quanta Biosciences, Beverly, MA) according to the manufacturer's instructions. A total of 20 µl reaction system was prepared for quantitative RT-PCR using perfecta SYBR Green Supermix (#95054; Quanta Biosciences). All reactions were run in triplicate, using a real-time PCR system (CFX96; BioRad), and the specificity of every reaction was determined using melting curve analysis. The expression level of target genes was normalized to *β-actin* or *GAPDH* (see figures and figure legends) and calculated using the 2^{-C_t} method. Full list of primers used is provided in Supplementary Methods.

Western blot.

Details in Supplementary Methods.

Cells proliferation assay.

Ki-67 labeling assay and cell number counting assay were used to assess the proliferation capacity of BMECs after myelin debris engulfment. Detailed information in Supplementary Methods.

In vivo Matrigel plug angiogenesis assay.

Matrigel plug angiogenesis assay was performed with a method modified from our previous publication⁵⁹. Briefly, 8×10^5 primary myelin-ECs or naïve-ECs were mixed with 100 μ l of precooled Matrigel solution. The mixtures were subcutaneously injected into mice. Myelin-ECs, naïve-ECs and PBS (as blank control) were injected in the same mouse at different sites. After implantation for 7 d, the Matrigel plugs were excised, immediately photographed with a MVX10 Macro Zoom microscope (Olympus), and then regular tissue histology and immunofluorescent staining for CD31 was used to label microvessels, whose density was analyzed and calculated as the percentage of CD31-positive area to the whole field.

BMDM ϕ adhesion on endothelial cells.

BDECs were seeded in 24-well plates and treated with or without 1 mg/ml CFSE-labeled myelin debris for 72 h to induce myelin-ECs. After removal of the myelin debris remnant in the culture, 3×10^5 BMDM ϕ were added to naïve-ECs and myelin-ECs monolayer. After 1 h adhesion, non-adhered BMDM ϕ were gently washed away with PBS five times. The adhered BMDM ϕ on endothelial cell monolayer were stained with Mac-2 antibody and imaged by phase contrast and fluorescent microscopy for visualization of the endothelial monolayer and Mac-2⁺ BMDM ϕ , respectively. The number of Mac-2⁺ BMDM ϕ that adhered on endothelial monolayer was counted and normalized to the number of endothelial cells as the percentage of BMDM ϕ adhesion onto endothelial monolayer. More information in Supplementary Methods.

BMDM ϕ chemotaxis toward endothelial cell supernatant.

A modified transwell assay was used to examine BMDM ϕ chemotaxis towards EC's supernatant. After BMECs engulf myelin debris for three days, they were quickly washed with PBS three times. This step gets rid of most remaining non-engulfed myelin debris in ECs. After the washes, myelin-ECs were cultured for 24 h in fresh culture media, followed by collection of cell culture supernatant from the myelin-ECs. The cell culture supernatant was clarified by centrifugation to remove any remaining myelin debris. Then, the BMEC supernatant was placed in the bottom chamber of the transwell, and BMDM ϕ were seeded on the upper chamber. After chemotaxis for 6 hr, migrated BMDM ϕ on the lower side of the membrane were stained with crystal violet (Alfa Aesar).

Astrocyte–endothelial cell co-culture assay.

Primary astrocytes were isolated from C57BL/6 mice aged between 2 d and 3 d⁶⁰. For astrocyte–endothelial cell co-culture, we used a transwell system in which astrocytes were seeded in the lower chamber and BMECs were seeded in the upper chamber. Detailed information in Supplementary Methods.

In vitro endothelial-to-mesenchymal transition assay.

Myelin debris (1 mg/ml) was added to BMECs for 1, 3, 5, 7, and 10 d, and culture medium was changed every 3 d, followed by morphological observation of spindle-shape cells. BMECs were considered spindle shaped when the diameter at their longest axis was 1.5-fold

greater than the average diameter of untreated cobblestone BMECs. Detailed information in Supplementary Methods.

Spinal cord BMEC injection.

Myelin-ECs were generated by treatment with myelin debris for 72 h followed by washing to remove non-ingested myelin debris. Both control BMECs (naïve-ECs) and myelin-ECs were fluorescently labeled after incubation for 1 h with 50 μ M CFSE in DMEM without serum and washed once with PBS containing 100 mM glycine and twice with PBS. The cells were trypsinized and resuspended in cold PBS. The CFSE signals in naïve-ECs and myelin-ECs were confirmed after labeling. The fluorescence intensity of CFSE in myelin-ECs were much more rapidly diluted than naïve-ECs after subculture (data not shown), probably due to robust proliferation of myelin-ECs, as shown in Fig. 5. BMECs at 1×10^5 in 0.5 μ l were injected in T10 spinal cords of normal mice using a 33 gauge needle attached to Hamilton syringe. 0.5 μ l PBS injection was used as blank control. Myelin debris in 0.5 μ l injection was included as a control to illustrate whether myelin debris, without endothelial cell uptake, could elicit any response compared with myelin-EC injection. ECs cultured with myelin debris in the presence of 70 °C heated serum (IgG inactivated), which demonstrated that myelin debris contacted endothelial cells without being internalized, were included to illustrate whether blocking endothelial cell myelin uptake could abrogate any in vivo spinal cord responses induced by myelin-ECs. The spinal cords were minimally exposed to avoid strong mechanical injury that may cause any unfavorable interference with cell injection. After 7 d of injection, mice were anaesthetized and perfused, then spinal cords were collected for regular histology and immunostaining.

Statistical analysis.

Data distribution was assumed to be normal, but this was not formally tested. The statistical significance of the difference between control and experimental groups was determined by two-sided Student's *t* test, unless otherwise indicated, using Prism 7 (GraphPad, San Diego, CA). Differences were considered statistically significant when $P < 0.05$. * $P < 0.05$, ** $P < 0.01$, <0.001 and **** $P < 0.0001$, as shown in figures and figure legends. Data are shown as mean $\pm$ s.e.m. unless otherwise indicated.

Reporting Summary.

Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

Data availability

All data supporting the findings of the current study are available from the corresponding authors upon reasonable request.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1 | Engulfment of myelin debris by spinal microvessels in SCI and EAE mouse models and endothelial cell-induced microvessel-like structures in vitro.
a–c, Internalization of myelin debris (MBP staining, green) by microvessels (CD31 staining, red) in normal spinal cords from uninjured mice (**a**) and spinal cords from injured mice at 1 week after SCI (**b**) or 1 week of EAE (**c**). The *xy*, *xz* and *yz* views (**a'–c'**) show myelin debris internalization by microvessels in SCI and EAE spinal cords. **d**, Quantification of myelin-containing microvessels in normal, SCI and EAE spinal cords. Myelin uninjured and injured regions were classified in SCI and classification details are described in Methods. Data are shown as means $\pm$ s.e.m.; $n = 4$ mice in SCI, $n = 3$ mice in EAE. 1-d SCI, $P = 0.779$ (NS, not significant); 3-d SCI, $*P = 0.0111$; 5-d SCI, $**P = 0.0088$; 7-d SCI, $*P = 0.0121$; 7-d EAE, $**P = 0.0076$ by paired two-sided Student's *t* test. **e, e'**, Detection of neutral lipids (ORO, red) in spinal microvessels from mice after 2 weeks of SCI. **e'**, 3D reconstruction of microvessel (CD31, green) shows accumulation of ORO⁺ lipids inside microvessels. Representative images of three independent mice. **f, f'**, Distribution of CFSE-labeled myelin

debris (green) in primary BMEC-assembled microvessel-like structures (CD31, red) on Matrigel that were incubated with myelin debris for 72 h. The *xy* and *yz* views of one region of interest show myelin debris approaching to but not contacting microvessel-like structures (**f'**1) or starting to touch (**f'**2) or entering (**f'**3) microvessel-like structures. Representative images of two independent staining experiments. Scale bar, 50 μm (**a**, **b**, **c**), 20 μm (**a'**–**c'**), 10 μm (**e**), 50 μm (**f**), 5 μm (**f'**).

Fig. 2 | In vitro engulfment of myelin debris by brain microvascular endothelial cells (BMECs).

a, Representative confocal images showing engulfment of CFSE-labeled myelin debris (green) by primary BMECs (CD31, red) after exposure to myelin debris for the indicated time points. Scale bar, 20 μ m. **b**, Flow cytometry detection of the percentage of primary BMECs with or without CFSE-myelin debris treatment for 72 h. Data are shown as means $\pm$ s.e.m. ($n = 3$ independent cell cultures). **** $P = 0.00009$ by unpaired two-sided Student's t test. **c**, ELISA detection of intracellular MBP in primary BMECs treated with or without myelin debris for 72 h. Data are shown as means $\pm$ s.e.m. ($n = 3$ independent cell cultures). * $P = 0.0144$ by unpaired two-sided Student's t test. **d**, Representative confocal images

showing engulfment of CFSE-myelin debris (green) by bEnd.3 cell line (CD31, red) after myelin debris treatment for the indicated time points. Scale bar, 20 μ m. **e**, FACS detection of the percentage of bEnd.3 cell line treated with or without myelin debris at the indicated time points. Data are shown as means $\pm$ s.e.m. ($n = 3$ independent cell cultures). 24 h vs. 0 h, **** $P = 0.00007$; 48 h vs. 0 h, *** $P = 0.0004$; 72 h vs. 0 h **** $P < 0.00001$; 96 h vs. 0 h, **** $P < 0.00001$; 96 h vs. 72 h, $P = 0.1308$ (NS) by unpaired two-sided Student's t test. **f**, ELISA detection of intracellular MBP in bEnd.3 cell line treated with or without myelin debris at the indicated time points. Data are shown as means $\pm$ s.e.m. ($n = 3$ independent cell cultures). 24 h vs. 0 h, $P = 0.1939$ (NS); 48 h vs. 0 h, * $P = 0.0173$; 72 h vs. 0 h, *** $P = 0.0006$; 96 h vs. 0 h, *** $P = 0.0008$; 96 h vs. 72 h, $P = 0.1884$ (NS) by unpaired two-sided Student's t test. **g**, Representative confocal images showing CFSE-myelin debris uptake by BMECs pretreated with control IgG, CR3 or Mac-2 neutralizing antibodies. ECs, endothelial cells. Scale bar, 20 μ m. **h**, Representative images showing BMEC engulfment of CFSE-myelin debris in serum heated at 56 $^{\circ}$ C for 20 min (to inactivate C3, left) or at 70 $^{\circ}$ C for 20 min (to inactivate IgG, middle) and IgG-opsonized myelin debris in IgG inactivated serum (right). Scale bar, 20 μ m. **i**, Corresponding quantification was analyzed by ELISA detection of intracellular MBP in BMECs treated with myelin debris, IgG-opsonized myelin debris for 72 h in the medium containing normal serum, C3-inactivated serum, no serum, IgG-inactivated serum or IgG-supplemented serum, respectively. Data are shown as means $\pm$ s.e.m. ($n = 3$ independent cell cultures). **** $P = 0.0005$; ** $P = 0.0011$ were calculated in comparison to normal serum group by unpaired two-sided Student's t test. Among no-serum groups, IgG-opsonized myelin vs. control, ### $P = 0.0001$; IgG supplementation vs. control, ### $P = 0.00003$. Among IgG-inactivated groups, IgG-opsonized myelin vs. control, ## $P = 0.0013$; IgG supplementation vs. control, ## $P = 0.0029$.

Fig. 3 |. Transcriptome comparison of naïve-BMECs and myelin-BMECs.

a, Heat map comparing naïve-BMECs and myelin-BMECs for the top 50 most changed genes as determined by pairwise comparisons using DESeq2. Two biological replicates were used for RNA sequencing analysis. Each biological replicate had a corresponding technical replicate. **b,c**, The upregulated and downregulated genes enriched in different groups in myelin-BMECs. Values show log₂-fold changes. Adjusted *P* values are in Supplemental Table 1 and Supplementary RNA sequencing Dataset. *P* values were corrected using a Benjamini-Hochberg adjustment method to control for false discovery rate. **d,e**, Quantitative RT-PCR analysis of gene expression related to pro-fibrotic (**d**) and inflammatory responses (**e**) in naïve-BMECs and myelin-BMECs. The gene expression was normalized to *GAPDH* (**d**) and *ACTB* (**e**). Data are shown as means $\pm$ s.e.m. (*n* = 3 biologically independent replicates). Collagen 1 α 2, ****P* = 0.0001; Collagen 1 α 1, ***P* = 0.0015; Collagen 5 α 2, ***P*

= 0.0028; MCP-1, **** $P < 0.00001$; IL-6, **** $P = 0.00007$; IL-4, *** $P = 0.0003$; iNOS, **** $P = 0.00005$ by unpaired two-sided Student's t test.

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Fig. 4 | Engulfed myelin debris is delivered through autophagosomes to lysosomes for degradation to lipids in BMECs.

a,a', Lysosomes stained with Lysotracker Red dye (red) in BMECs treated with or without CFSE-myelin debris (green) for 72 h. The zoomed images (**a'**) show the detailed size and spatial relationship of lysosomes and engulfed myelin debris in naïve-BMECs and myelin-BMECs. Arrowheads indicate lysosomes containing no myelin debris; arrows indicate lysosomes containing engulfed myelin debris. Scale bar, 10 μ m (**a**), 1 μ m (**a'**). **b**, Quantification of the lysosome size in naïve-BMECs and myelin-BMECs. Each dot indicates lysotracker red-positive puncta. Data are shown as means $\pm$ s.e.m. ($n = 142$ for naïve ECs, $n = 174$ for lysosomes without myelin, $n = 240$ for lysosomes with myelin from at least ten cells in three biologically independent replicates). **** $P < 0.00001$ by unpaired Student's t test. **c**, Representative confocal images and 3D heat map of colocalization between CFSE-myelin puncta (green) and LC3⁺ or GABARAP⁺ autophagosomes (red), between myelin puncta (green) and Rab5⁺ early endosomes (red) or Rab7⁺ late endosomes

(red). Scale bar, 1 μm . **d**, Colocalization analysis between myelin debris and other vesicular puncta as indicated. Data are shown as means $\pm$ s.e.m. from at least ten images in three biological replicates. **** $P=0.00003$ for LC3 vs. Rab5; *** $P=0.00048$ for LC3 vs. Rab7; **** $P=0.00005$ for GABARAP vs. Rab5; *** $P=0.00032$ for GABARAP vs. Rab7 by unpaired two-sided Student's t test. **e**, Quantification of LC3⁺ puncta per cell in BMECs cultured with 5% serum (fed), after starvation for 6 h (starved) and after treatment with myelin debris for 72 h (myelin ECs). Data are shown as means $\pm$ s.e.m. from at least three independent experiments ($n=3$ for fed group, $n=4$ each for starved and myelin debris groups). Starved vs. fed, ** $P=0.009$; myelin debris vs. fed, ** $P=0.0029$ by unpaired two-sided Student's t test. **f**, Immunoblot and densitometry quantification for LC3 and p62 in BMECs treated with myelin debris for 0 h, 24 h and 72 h. Data are shown as means $\pm$ s.e.m. from three independent immunoblots. LC3-II/LC3-I, * $P=0.0334$; p62/GAPDH, * $P=0.0301$ by unpaired two-sided Student's t test. Uncropped blots are in Supplementary Fig. 13. **g**, Immunoblot for Atg5 in the indicated Atg5^{-/-} BMEC lines generated by CRISPR-Cas9 technique. This initial western blot screening was performed once, yielding three lines (#4, 6, 8) that are negative for Atg5 protein expression. Uncropped blots are in Supplementary Fig. 13. **h**, Immunostaining of wild-type BMECs and #4 Atg5^{-/-} BMECs for anti-LC3 (green) in HBSS starvation-induced autophagy. CD31 (red) and Hoechst (blue) staining labels endothelial cells and nuclei, respectively. This LC3 staining verification was performed twice. Scale bar, 20 μm . **i**, Immunoblot for LC3-I/LC3-II conversion and p62 in #4 Atg5^{-/-} BMECs. This verification was performed once after initial screening in **g**. Uncropped blots are in Supplementary Fig. 13. **j**, Myelin degradation into neutral lipids (stained by ORO) in myelin-laden Atg5^{-/-} BMECs and wild-type (WT) BMECs treated with 10 μM chloroquine (24 h), 1 μM rapamycin (24 h). Scale bar, 20 μm . **k**, Quantification of ORO⁺ lipids in **j** as well as in myelin-BMECs after 48 h treatment with 1 mM 3-MA, 24 h culture in 0% FBS medium (starvation). Data are shown as means $\pm$ s.e.m. from three independent experiments. Atg5^{-/-}, ** $P=0.0075$; 3-MA, ** $P=0.0073$; chloroquine (chloro), ** $P=0.006$; rapamycin (rapa), *** $P=0.0005$; starved, * $P=0.0178$ were calculated in comparison with WT by unpaired two-sided Student's t test.

Fig. 5 | Myelin debris uptake contributes to angiogenesis in SCI and EAE.

a, Microvessels (CD31, red) in three different regions, classified as uninjured, marginal and injured regions (see Methods for detailed classification) in an injured spinal cord from 1-week SCI mouse. Scale bar, 500 μm ; 50 μm in zoomed images. **b**, Corresponding quantification of microvessel diameter in the normal spinal cord, uninjured region, marginal regions and injured regions from mice at 1, 4, 6, 8 and 10 weeks after SCI. Data are shown as means $\pm$ s.d. The number of mice analyzed is: normal ($n = 5$); 1-week ($n = 5$); 4-week ($n = 4$); 6-week ($n = 4$); 8-week ($n = 4$); 10-week ($n = 3$). **c**, Immunostaining for Ki-67 (green) and CD31 (red) in normal spinal cord from uninjured mice and injured spinal cord from 7-day SCI mice. The zoomed images showed the Ki-67⁺ microvessels. Arrowheads indicate Ki-67⁺ endothelial cells. Scale bar, 50 μm (upper images), 10 μm (lower images). **c'**, Corresponding quantification of Ki-67⁺ endothelial cells in normal spinal cords from uninjured mice and injured spinal cords from SCI mice. Data are shown as means $\pm$ s.e.m.

Normal mice ($n = 4$) and SCI mice ($n = 5$). $**P = 0.0029$ by unpaired two-sided Student's t test. **d**, Microvessels (CD31, red) and myelin (MBP, green) staining in 15-day EAE spinal cord. Arrowheads indicate enlarged microvessels in demyelinated regions (MBP negative). Scale bar, 50 μm ; 20 μm in zoomed images to the right. **d'**, Quantification of the microvessel diameter in non-demyelinated and demyelinated regions from 7-day and 15-day EAE spinal cords. Data are shown as means $\pm$ s.e.m. from 3 mice. 7-day, $*P = 0.0107$; 15-day, $*P = 0.0139$ by paired two-sided Student's t test. **e**, Immunostaining for Ki-67 (green) and CD31 (red) in spinal cord from 15 day EAE mouse. Arrowheads indicate Ki-67⁺ endothelial cells on microvessels. Scale bar, 20 μm . **f**, Quantification of Ki-67⁺ cells in primary BMECs and bEnd.3. cell line treated with myelin debris for 72 h or the indicated time points. Data are shown as means $\pm$ s.e.m. Naïve-ECs ($n = 4$ independent cultures), myelin-ECs ($n = 3$ independent cultures). Primary BMECs, $*P = 0.026$. bEnd.3. cell line, 72 h, $*P = 0.0226$; 96 h, $*P = 0.0118$; 120 h, $***P = 0.001$ by unpaired two-sided Student's t test. **g**, Number of BMECs treated with myelin debris for the indicated time points. Data are shown as means $\pm$ s.e.m. from three independent assays. 72 h, $**P = 0.0026$; 96 h, $****P = 0.00001$; 120 h, $**P = 0.0079$ by unpaired two-sided Student's t test. **h**, Gross images of Matrigel plugs injected subcutaneously with PBS, naïve-ECs and myelin-ECs in normal mice. Insets are CD31 immunostaining of Matrigel slices. Scale bar in insets, 20 μm . The bottom shows the corresponding quantification of CD31⁺ microvessels in each Matrigel plug. Data are shown as means $\pm$ s.e.m. from three independent Matrigel plugs. Myelin-ECs vs naïve-ECs, $***P = 0.0002$ by unpaired two-sided Student's t test. **i**, Cell numbers of wild-type or Atg5^{-/-} BMECs with or without myelin debris treatment for 72 h. Data are shown as means $\pm$ s.e.m. from three assays. $*P = 0.0401$ by unpaired two-sided Student's t test. **j**, Quantitative RT-PCR analysis of *VEGF* expression in naïve-BMECs and myelin-ECs. Gene expression was normalized to *GAPDH*. Data are shown as means $\pm$ s.e.m. from three biologically independent replicates. $****P < 0.00001$ by unpaired two-sided Student's t test. **k**, Cell number of BMECs treated with myelin debris in the presence of control IgG or neutralizing VEGF antibody (20 $\mu\text{g}/\text{ml}$, 72 h). Data are shown as means $\pm$ s.e.m. ($n = 3$ independent cultures). $P = 0.61$ (NS); $*P = 0.0184$ by unpaired two-sided Student's t test.

Fig. 6 | Myelin debris uptake induces endothelial inflammation leading to BMDMφ infiltration. **a,a'**, Representative confocal images (**a**) and 3D reconstructed images (**a'**) showing the spatiotemporal distribution of bone marrow-derived GFP⁺ cells (GFP⁺ BMDCs) and CD31⁺ microvessels (red) in normal and injured spinal cords from GFP⁺ bone marrow chimeric mice at 3 d, 1 week and 2 weeks after SCI. Scale bar, 20 μm. Quantification data is shown in Supplemental Fig. 12a. **b**, Representative confocal images and 3D reconstructed images showing the spatial distribution of Iba-1⁺ cells relative to microvessels (red) in 1-week EAE spinal cord. Scale bar, 20 μm. Quantification data are shown in Supplementary Fig. 12b. **c**, Representative images showing adhered BMDMφ (Mac-2, red) on the monolayer of naïve-ECs or myelin-ECs (DIC imaging, grey). Scale bar, 100 μm. **c'**, Corresponding quantification of adhered BMDMφ on BMECs in (c) and Atg5^{-/-} BMECs as well as wild-type (WT) BMECs cultured with 70 °C heated serum (that is, no IgG or IgG-inactivated serum), the value was shown as the percentage of adhered BMDMφ relative to the

underlying BMECs. Data are presented as means $\pm$ s.e.m. ($n = 3$ independent cultures). $**P = 0.0023$; $##P = 0.0072$; $\#P = 0.0129$ by unpaired two-sided Student's t test. **d**, Immunoblot and quantification of VCAM-1 expression in BMECs treated with myelin debris for the indicated time points. Quantification of VCAM-1 expression level was determined by densitometry analysis relative to GAPDH. Data are presented as means $\pm$ s.e.m. ($n = 3$ independent immunoblots). 1 d, $*P = 0.024$; 2 d, $***P = 0.0008$; 3 d, $**P = 0.0013$; 4 d, $**P = 0.0011$; 5 d, $**P = 0.0012$ by unpaired two-sided Student's t test. Uncropped blots are in Supplementary Fig. 13. **e**, Images of migrated BMDM ϕ (violet crystal staining) toward conditioned media from ECs. BMDM ϕ were added to the upper chamber and allowed to migrate through the membrane into the lower chamber containing conditioned media from naïve-ECs or myelin-ECs. This assay was performed twice with similar results. **f**, ELISA detection for chemokine MCP-1 secreted by naïve-ECs or myelin-ECs in primary endothelial cell cultures (left) or cell line (right). Data are shown as means $\pm$ s.e.m.; $n = 3$ independent cell cultures. $***P = 0.0006$; $**P = 0.0029$ by unpaired two-sided Student's t test. **g**, Quantitative RT-PCR analysis of *IL-6* in BMDM ϕ treated with conditioned media from naïve-ECs or myelin-ECs. Gene expression was normalized to *Actb*. Data are presented as means $\pm$ s.e.m. ($n = 3$ biologically independent replicates). $***P = 0.0002$ by unpaired two-sided Student's t test. **h**, Immunostaining of macrophages/microglia (Iba-1, green) and astrocytes (GFAP, red) in normal spinal cords injected with CFSE-labeled naïve-ECs or myelin-ECs (white). Quantification data are shown in Supplementary Fig. 11e,f. Scale bar, 100 μ m, 10 μ m (inset images).

Fig. 7 | Myelin debris engulment promotes endothelial deposition of profibrotic components. **a–c**, Immunostaining for collagen I (green) and CD31 (red) in normal spinal cords (**a**) and in the lesion cores from mice at 6 weeks after SCI (**b**) and in 15-day EAE spinal cord (**c**). Scale bar, 500 μ m (upper images), 20 μ m (lower zoomed images). Representative images of three independent mice. **d–f**, Immunostaining for fibronectin (green) and CD31 (red) in normal spinal cords (**d**) and in the lesion cores from mice at 6 weeks after SCI (**e**) and in 15-day EAE spinal cord (**f**). Scale bar, 500 μ m (upper images), 20 μ m (lower zoomed images). Representative images of three independent mice. **g**, Immunostaining of collagen I (green) and CD31 (red) in BMECs treated with or without CFSE-myelin debris (pseudo-white) for 10 days or treatment with mouse recombinant TGF- β 1 (10 ng/ml) for 3 days. Scale bar, 20 μ m. (**g'**) Quantification of fluorescent intensity of collagen I in **g**. Data are shown as means $\pm$ s.e.m. ($n = 3$). Myelin vs. control, $*P = 0.0301$; TGF- β 1 vs. control, $*P = 0.0159$ by two-sided Student's t test. **h**, Immunostaining of collagen I (green) in BMEC-induced

microvessel-like structures (CD31, red) treated with or without CFSE-myelin debris (pseudo-white) for 72 h. Scale bar, 100 μ m. This staining was performed twice with similar results. **i**, Immunoblot for fibronectin in BMECs at baseline or after treatment with myelin debris for the indicated time points. Treatment with TGF- β 1 (10 ng/ml) for 5 d was used as positive control. Corresponding quantification of protein levels was determined by densitometry analysis relative to GAPDH or tubulin, as shown at the bottom of each blot. The immunoblots were performed twice with similar results. Uncropped blot is in Supplementary Fig. 13.

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Fig. 8 | Myelin debris engulfment induces endothelial-to-mesenchymal transition.

a, Phase contrast images of WT and Atg5^{-/-} BMECs with the indicated treatment: TGF- β 1 (10 ng/ml, 3 d), myelin debris (1 mg/ml, 10 d), myelin + pan-TGF- β neutralizing antibody (20 μ g/ml, 10 d). The insets show the magnified views of cell morphology. **b**, Immunostaining of α -SMA (green) and CD31 (red) of WT and Atg5^{-/-} BMECs with the treatment indicated above; myelin debris is shown in pseudo-white. The zoomed images below show detailed immunostainings of α -SMA, CD31 and engulfed myelin debris. Scale bar, 20 μ m (upper images), 10 μ m (lower images). **c**, Quantification of spindle-shaped BMECs. The left diagram shows the criteria for spindle-shape cells. Data are shown as means $\pm$ s.e.m. ($n = 3$ independent assays). *** $P = 0.0008$, ** $P = 0.0027$ vs. control, ## $P = 0.0029$, ### $P = 0.001$ vs. WT myelin group; analysis by unpaired two-sided Student's t test. **d**, Quantification of α -SMA⁺CD31⁺ BMECs. Data are shown as means $\pm$ s.e.m. ($n = 3$ independent assays). TGF- β 1 vs. control, ** $P = 0.0015$; myelin vs. control, ** $P = 0.0063$;

myelin + TGF- β antibody vs. myelin, $^{##}P = 0.0094$; myelin + Atg5 $^{-/-}$ vs. myelin + wild type, $^{##}P = 0.0063$, analysis by unpaired two-sided Student's t test. **e**, Quantitative RT-PCR analysis of *aSMA* expression in naïve-ECs and myelin-ECs. Gene expression was normalized to *GAPDH*. Data are shown as means $\pm$ s.e.m. from three biologically independent replicates. $^{**}P = 0.0057$ by unpaired two-sided Student's t test. **f**, Immunoblot for α -SMA in BMECs at baseline or treatment with myelin debris for the indicated time points. Treatment with TGF- β 1 (10 ng/ml) for 5 d was used as positive control. Corresponding quantification of protein levels were determined by densitometry analysis relative to GAPDH or tubulin, as shown at the bottom of each blot. The immunoblots were performed twice with similar results. Uncropped blot is in Supplementary Fig. 13. **g**, Quantitative RT-PCR analysis of gene expression of *Tgfb1* in naïve-ECs and myelin-ECs. Data are shown as means $\pm$ s.e.m. from three biologically independent replicates. Gene expression was normalized to *Actb*. $^{***}P = 0.0002$ by unpaired two-sided Student's t test. **h**, Immunostaining for α -SMA (green) and CD31 (red) in normal spinal cords and in the lesion cores from mice at 6 weeks after SCI and 15 d of EAE. The staining was performed in three mice with similar results. Scale bar, 20 μ m (images to the left), 5 μ m (zoomed images to the right).