Supplemental information

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Innate and adaptive immune cells: annotation and pathway analysis

To better characterize the innate and adaptive immune cell subtypes, we first selected clusters annotated as immune cells (clusters 5,10, and 17, with high gene expression of CSF1R, C3, CD74, and/or RUNX1), and lymphocytes (cluster 16, with high expression of PTPRC, CD2, and SKAP1) (Fig. 1c). Unsupervised clustering of these 5,838 nuclei created a new map of 11 transcriptionally distinct cell populations (Fig. 1g and 2a–b). The list of the top 100 differentially expressed genes for each cluster is in Supplementary Table 4, and for each lesion location in Supplementary Table 12. Tissue validation is shown in Fig. 2 and 3 and Extended Fig. 4 and 7.

Leveraging markers previously reported to distinguish microglia from peripherally derived monocytes/macrophages, we quantified the expression of SALL1[1] and TMEM119[2] (previously reported microglial markers) as Z-scores for each cluster relative to all immune cells. Populations hereafter annotated as "microglia" showed positive Z-scores, whereas monocytes/mature dendritic cells, macrophages, and lymphocytes had negative Z-scores. This approach might underestimate the number of some activated microglia. Indeed, a clear phenotypic distinction between resident and CNS-invading peripheral monocytic cells is currently the object of an intense scientific debate, as demonstrated by a recent study[3] showing that peripheral monocytes, upon entry into a CNS, can start to express some typical microglial markers.

The overall interpretation of our results in relation to tissue type is reported in the main text. Hereafter we provide a detailed annotation and description of all immune cell subclusters:

- Cluster 0 was annotated as "homeostatic microglia" based on previously reported phenotype-defining genes (P2RY12, CXC3CR1, CSF1R, SRGAP2).[4-7] We selected P2YR12 for further identification of homeostatic microglia in tissue. As expected, we found downregulation of P2YR12 at the chronic active lesion edge in comparison to the periplaque white matter (Extended Fig. 4 insets 3–4).
- Clusters 1 and 8 were both annotated as "microglia inflamed in MS (MIMS)" based on a transcriptional profile (including TREM2, APOE, LPL, CD68, CD9, CD74, GNR, TYROBP, TIMP2, SSP1, CTSZ, CTSB, FTH1, C1Q) overlapping with that described in other neurodegenerative diseases and disease models.[4-7] However, in MS tissue, two distinct subpopulations could be defined:
    - Cluster 1 (annotated as "MIMS-foamy") enriched for foam cell differentiation and lipid storage (MERTK, LPL, ABCA1, PPARG, MSR1, IL18, ITGAV), response to lipoprotein particles (LPL, APOE, ABCA1, PPARG, CD81), lysosome (LIPA, ASAH1, AP1B1, CTSB, PSAP, PPT1,

CTSL), and regulation of inflammatory response (TREM2, APOE, LPL, NUPR1, PLA2G7, CEBPA, PPARG, CD81, PLD3, SERPINE1, HGF, IL18). Pathway analysis is shown in Extended Fig. 5a. In tissue, this cluster was identified at the chronic active lesion edge by co-expression of IBA1 CD68, MHCII, PPARG, $FTL^{low}$ (Fig. 2f and Extended Fig. 4 inset 7).

- Cluster 8 (annotated as "MIMS-iron") enriched for ribosome (63 ribosomal proteins within the first 100 top differentially expressed genes), reflecting their strongly activated state. These cells also upregulated ferritin complex (FTL, FTH1), MHC class II protein complex (HLA-DRA, HLA-DPA1, CD74), and complement component C1 complex (C1QA, C1QB). Pathway analysis is shown in Extended Data Fig. 5b. Among immune cells, this was the population showing the highest expression of IL1B (Z-score=1.96). When directly compared to MIMS-foamy (volcano plot, Fig. 2c), MIMS-iron showed higher expression of MHC II-related and inflammatory markers, including SOD1, iron-related genes (FTH1, FTL), and complement component C1 complex genes (C1QA, C1QB, C1QC). In tissue, this cluster was identified at the chronic active lesion edge by co-expression of IBA1 CD68 PPARG MHCII and $FTL^{high}$ (Fig. 2f, Fig. 4c and Extended Fig. 4 inset 7 and 7b).

The identification of two distinct MIMS subpopulations (prevalently myelin-laden vs. more inflammatory iron-laden) is consistent with previous in vitro work showing that iron uptake is relatively limited in myelin-phagocytosing human macrophages and vice versa.[8] Of note, the recently described lipid-droplet-accumulating microglia (LDAM)[9] gene profile in aged mice was not associated with any specific clusters, although one of the markers, CD63, was upregulated in both MIMS clusters, especially in MIMS-iron (Z-score=2.5). Interestingly, in MS brain tissue, we identified the co-presence of early (LFB+, blue) and late (PAS+, purple, lipofuscin-laden[10]) myelin degradation products within phagocytes (potentially two stages of foam cells) at the chronic active lesion edge (Extended Data Fig. 1c).

- Clusters 2 and 6 were annotated as "Monocytes/monocyte-derived dendritic cells (Mono/moDC)" based on the upregulation of CD83 and relatively lower expression of SALL1 and TMEM119 compared to all immune cells (negative Z-scores). Refined detailed annotation of peripheral immune cells is presented in Supplementary Table 8. In tissue, we found CD83 positive cells mixed with CD68-IBA1 phagocytes at the chronic active lesion edge (Extended Fig. 4 inset 8). These clusters were exclusively seen in MS tissue.
  - Cluster 2 (annotated only as "Mono/moDC") enriched for response to unfolded protein and stress (HSPH1, BAG3, HSP90AA1, HSPD1, DNAJB1, HSPB1, SERPINH1, HSP90AB1, HSPA6, HSPA4, DNAJA1, HSPA1A, PPP1R15A, HSPA4L, HSPA8), antigen processing and presentation (HSP90AA1, HSP90AB1, HSPA6, HSPA4, HSPA1A, HSPA8), MHC class II protein complex (HSP90AA1, HSP90AB1, HSPA8), and NLRP3 inflammasome (HSP90AB1, APP, NFKB1).
  - Cluster 6 (annotated as "Mono/moDC hypoxia") enriched for the hypoxia inducible factor (HIF)-1a signalling pathway (HIF1A, VEGFA, HK2, TFRC, PDK1, PGK1, HMOX1, NFKB1), cellular response to hypoxia (VEGFA, HIF1A, BNIP3L, ERO1A, PDK1, BNIP3, HILPDA, PGK1,

HMOX1), and ferroptosis (TFRC, ACSL1, HMOX1, ACSL4). This cluster might represent the HIF-1a-dependent hypoxic dendritic cells exposed to a low oxygen environment.[11-14]

- Cluster 3 was annotated as "microglia/macrophages with oxidoreductase activity" due to their intermediate SALL1 and TMEM119 expression and to the pathway analysis terms. These cells enriched for oxidoreductase activity (KDM5A, TBXAS1, MT-CO2, CTBP2, MT-CO3, GAPDH, MT-ND3, ALOX5, MT-ND5, MT-ND4, OXR1, MT-ND1, MT-ND2, DHFR), respiratory electron transport chain (MT-CO2, MT-CO3, MT-ND3, MT-ND5, MT-ND4, MT-ND1, MT-ND2), oxidative phosphorylation (MT-CO2, MT-CO3, MT-ND3, MT-ND5, MT-ND4, MT-ND1, MT-ND2), and NADH dehydrogenase complex (MT-ND3, MT-ND5, MT-ND4, MT-ND1, MT-ND2).
- Cluster 4 was annotated as "perinodal microglia" due to enrichment for genes related to axon (IL1RAPL1, PCDH9, NCAM2, MAP7, SPOCK1, KIRREL3, PTPRK, DNM3, UNC5C, DLG2, NFASC, GRM3, APBB2, DSCAML1, CNTN2, MAPT, COBL, ANK3, CADM2, ANK2), node of Ranvier (SPOCK1, NFASC, CNTN2, ANK3), and cell adhesion molecules and cell-cell junction (NCAM2, NRXN3, NCAM1, NLGN1, NFASC, CNTN2, JAM3). Although we cannot exclude some misclassification, this microglia cluster showed the highest level of myelin transcripts among all microglial cell types. Myelin transcripts have been identified in microglia nuclei in a previous snRNA-seq study, a finding interpreted to reflect uptake of phagocytized myelin transcripts in the nuclear-perinuclear area[15] from distal oligodendrocytes branches.[16,17] This cluster was seen in both MS and control tissue. The pathway analysis and high level myelin transcripts might suggest specific localization of this type of microglia at the level of the node of Ranvier and a role in adaptive myelination.[18]
- Cluster 5 was annotated as "macrophages" based on the differential gene expression of MRC1, CD163, CD163L1, MSR1, STAB, LYVE1. Genes enriched for terms related to endocytosis (CD163, MRC1, CD163L1, MCTP1, FCHSD2, FCGR2B, EPS15, STAB1, FCHO2, LRRK2, RAB20, MSR1, ITSN1, HSP90B1) and endosome (MRC1, MCTP1, SLC9A9, FCHSD2, IL15, TBC1D14, NRP1, EPS15, KIF16B, LRRK2, RAB20, MYO5A, SNX6, ITSN1, LITAF). About 25% of the nuclei of this cluster were LYVE1-expressing perivascular macrophages. This population express also CLEC12A (z-score of 2.5 relative to other immune populations), a known macrophage marker.
- Cluster 7 was annotated as "T-cells" (CD2, CD8A, CCL5, SKAP1). They enriched for T-cell differentiation and T-cell activation (CAMK4, THEMIS, IL7R, BCL11B, TOX, GRAP2, ITK, CCL5, CD2, FYN, PRKCQ, LCK, RASGRP1, RUNX3, RIPOR2, PTPN22, RORA, CD44, CCND3), T-cell receptor signalling pathway (THEMIS, SKAP1, GRAP2, ITK, FYN, PRKCQ, LCK, CD247, PTPN22), regulation of cell-cell adhesion (SKAP1, IL7R, ETS1, GRAP2, CCL5, FYN, PRKCQ, LCK, RASGRP1, RUNX3, ITGA4, RIPOR2, PTPN22, CD44, ANK3), and positive interferon-gamma secretion (CD2,RASGRP1,PTPN22), protein kinase activity (CAMK4, ITK, CCL5, FYN, PRKCQ, LCK, PRKCH, ERN1, STK17B, STK17A, CCND3, CASK, STK39, TNIK, PRKCB, GRK5). As reported in Source Table 8, we only rarely observed CD4 T cells (central or effector memory) in our sample. At tissue validation, CD8 T cells and CD20 B cells were seen mostly within the perivascular spaces at the chronic active lesion edge (Extended Fig. 4 insets 1 and 2).
- Cluster 9 — a very small cluster of 113 nuclei — was annotated as "Other" due to the difficulty in defining its identity based on the gene expression and pathway analysis.

- Cluster 10 was annotated as "plasmablasts/plasma cells" (CD38, CD79A, IGHG, IGHA, IGHM). They enriched for B-cell activation (IGHG1, IGHG4, IGHA1, IGHM, MZB1, CD79A, IGLL5, CD38, BANK1, XBP1, BLC2, ITGA4, TPD52), immunoglobulin receptor binding and antigen binding (IGHG1, IGHG4, IGHA1, IGHM, JCHAIN), and protein processing in the endoplasmic reticulum (DERL3, SSR4, XBP1, ERN1, UBE2J1, SEC24D, RRBP1, DNAJC1, EIF2AK3, BCL2, EDEM1, SEL1L). In this cluster, CD20 (MS41A) was not expressed.

Since TSPO has been the most frequently implemented PET imaging target of neuroinflammation in vivo,[19] including of chronic active MS lesions,[20] we interrogated our snRNA-seq dataset to identify the cell clusters that express this gene at high levels. High TSPO nuclear expression was found in MIMS-iron followed by MIMS-foamy (Z-scores of 2.51 and 0.91, respectively, relative to all immune cells), AIMS (Z-scores of 2.6 relative to all astrocytes), and endothelial cells. In line with previous literature,[21] although TSPO is not specific to microglia, we confirm that TSPO is upregulated in the critical microglial and astrocytic inflammatory clusters seen at the edge of chronic active MS lesions and therefore remains a viable PET target of chronic inflammation in MS.

Oligodendrocyte lineage: annotation and pathway analysis

From the initial UMAP, we subclustered the 47,872 nuclei belonging to oligodendrocyte lineage (45,697 oligodendrocytes and 2,175 OPC, Fig. 1h). The list of the top 100 differentially expressed genes for each cluster is in Supplementary Table 5, and selected differentially expressed genes are shown in stacked violin plots in Extended Data Fig. 3b–c. OPC formed a separate cluster from oligodendrocytes in both the main clustering and the subclustering (cluster 5).

We identified 7 oligodendrocyte clusters. Based on the cluster tree map and clusters' frequency in the analysed brain tissues, we annotated most of these cells as "normal oligodendrocytes" (clusters 0, 1, 2, and 4 of the oligodendrocyte lineage subclustering, 38,651 nuclei). Only relatively few differentially expressed genes (<100) distinguished these clusters from one another. As expected, the pathway analysis included common enrichment terms such as structural constituent of myelin sheath, myelination, and axon ensheathment. Of note, oligodendrocyte cluster 4 upregulated genes involved in the interaction between axons and nodes of Ranvier (ANK3, OPALIN). Our analysis is consistent with Jäkel et al.'s[22] report and tissue validation of OPALIN+ oligodendrocyte clusters.

We annotated clusters 3, 6, and 7 as "stressed oligodendrocytes" (7,043 nuclei, 15% of all oligodendrocytes). Oligodendrocyte clusters 3 and 7 (6,038 nuclei, 13% of oligodendrocytes analysed) were exclusively seen in MS tissue, especially in the periplaque and at the edge of chronic active MS lesions. Oligodendrocyte cluster 3 (5,090 nuclei) enriched for unfolded protein binding, heat-shock protein binding (HSPD1, DNAJA4, DNAJB1, PTGES3, SERPINH1, ST13, HSP90AB1, HSPA1B, DNAJB6, HSPA6, HSP90AA1, TCP1, HSPA1A, HSPA9, HSPA8, DNAJA1), and ubiquitin protein ligase binding (UBC, UBB, CACYBP, JUN, UBE2B).[23,24] Of note, in the small oligodendrocyte cluster 7 (948 nuclei), we found overall downregulation of myelin proteins (PLP1, CNP, MAG, MOG) and upregulation of LINGO1, a

known negative regulator of myelination, whereas oligodendrocyte cluster 6 (1,005 nuclei) showed high expression of mitochondrial genes involved in oxidoreductase activity.

Subclustering of the 2,175 OPC nuclei (differential gene expression of PDFGRA, CSPG4, SOX6) from the initial UMAP (cluster 8) showed 8 distinct OPC clusters (Fig. 1i). The list of the top 100 differentially expressed genes for each cluster is in Supplementary Table 6. Hereafter the detailed annotation and description of all identified OPC clusters; the overall interpretation in relation to the pathological stages is reported in the main text:

- Clusters 0 and 2 were annotated as "normal white matter OPC" (914 nuclei) based on their presence and high frequency in the white matter of neurologically healthy control brain tissue. They enriched for channel activity (KCNIP4, KCND2, GRIK1, CNTNAP2, GRIK2, CACNG4, PDE4D, KCNQ3, KCNIP1, VWC2) and synaptic genes (HIP1, GSG1L, PCDH15, DAB1, PTPRT, KCND2, GRIK1, KCTD16, ANKS1B, SLC8A1, GRIK2, CACNG4, PDE7B, RIMS1, KCNQ3, PRKN, GPC6, DCC, ITGA8, PTPRO, RAB27B, NSF, VWC2, NLGN4Y, LRFN5), potentially suggesting direct contact and networking with other cell types.
- Cluster 1 was annotated as "stressed OPC" (448 nuclei). This cluster is specific to MS tissue and was seen in all pathological lesion stages analysed here. Cells upregulated cholesterol biosynthesis (HMGCS1, MSMO1, SQLE, FDFT1, HMGCR, INSIG1, SCD, ACAT2, SREBF2), unfolded protein response (HSP90A1, DNAJB1, HSPA1A, HSPA1B, HSPD1, PTGES3, TCP1, HSP90AB1, ST13, HSPA9), and regulation of response to stress (CAMK2D, FXR1, BCL6, CHORDC1, VEGFA, RNF168, UBR5, IFI16).
- Clusters 3 and 5 were annotated as "immunological OPC" (358 nuclei) for the upregulation of MHC class I and complement component C1 complex genes. We specifically saw upregulation of HLA-A, HLA-C, B2M, CD81, CD9, C1QL1, as well as ribosomal and synaptic genes. Inflammatory or "immunological" OPC have been recently described in mouse MS models[25,26] and MS tissue,[22] and have been hypothesized to contribute to antigen presentation within the CNS in both homeostasis and disease. In our dataset, they had higher frequency at the edge of chronic active lesions (23% of all OPC).
- Cluster 4 was annotated as "senescent OPC" (a total of 177 nuclei) for the upregulation of HMGB1, as recently described in brain tissue from progressive MS cases.[27] In line with previous data, this cluster was frequent especially in the core of the lesion (35% of all OPC).
- Cluster 6 was annotated as "myelinating OPC or oligodendrocytes" (a total of 112 nuclei) because of the high differential expression of myelin genes (MOBP, PLP1, MBP). Accordingly, they enriched for structural constituents of the myelin sheath (MOBP, PLP1, MBP), axon (GRM3, MAP7, LRP2, CNTN2, SHTN1, IQGAP1, KIRREL3, MYO1D, BIN1, MBP, AGTPBP1, CRYAB, PTPRK), lipid transporter activity (ABCA8, SLCO1A2, ABCA2, ABCA6, ATP8A1, OSBPL1A), and oligodendrocyte differentiation (CNTN2, DAAM2, LPAR1, ABCA2, MYRF, PLP1).
- Cluster 7 was annotated as "premyelinating OPC" (a total of 40 nuclei). In this population, we found upregulation of genes of the human oligodendrocyte module (BCAS1, KCNS3, GNB4, PPP1R16B), as described in Pol et al.,[28] and myelin proteins (PLP1). They enriched for semaphorin signalling (SEMA5A, SEMA5B, SEMA4D, SEMA6D) and axon guidance (FYN, EPHB1, ARHGAP35, NFASC, DSCAML1, TRIO, CYFIP2), suggesting possible engagement with unmyelinated or demyelinated

axons. The small percentage of premyelinating and myelinating OPC did not significantly differ across MS lesion pathological stages.

- Cluster 8 was a very small cluster of only 12 nuclei and possibly represents misclassified immune cells (RUNX1, MSR1, SSP1, FTL).

Astrocytes: annotation and pathway analysis

We analysed clusters initially annotated as astrocytes (8,211 nuclei, clusters 4, 9, 12, and 14 of the initial UMAP) based on known markers (SCL1A2, ALDH1L1, AQP4, LINC01088, VIM). Our analysis identified 9 transcriptionally distinct astrocyte populations from the initial UMAP (Fig.1j). The top 100 differentially expressed genes for each cluster are listed in Supplementary Table 7, and selected differentially expressed genes are shown in stacked violin plots in Extended Data Fig. 3a. Hereafter the detailed annotation and description of all clusters; the overall interpretation in relation to the pathological stages is reported in the main text:

- Clusters 0, 2, 3, and 8 were annotated as "nonreactive astrocytes" (5,326 nuclei) based on their high frequency in the white matter of neurologically healthy control brain tissue. They expressed genes related to transporter and ion channel activity (SLC1A2, SLC4A4, SLC6A11, RYR3, TRPM3, GABRB1, KCNQ3, SLC1A3, CACNB2, GPM6A, SLC35F1, SFXN5, SLC6A1, GRIA2, FAM155A, ATP1A2, GABRA2, GRAMD1C ,CACNA2D3), synaptic signalling (NRG3, STXBP5L, DGKB, ADCY8, KCND2, CD38, ADRA1A, CASK, NTNG1, LGI1, RNF19A, LAMA2, DLGAP1), axon (NRXN1, SLC1A2, NRP1, CADM2, TENM2, PCDH9, CTNNA2, KCNQ3, GRM3, ADGRL3, GPM6A, ALCAM, SLC6A1, EPHB1, GABRA2, OPHN1, CPEB4), and modulation of axon growth (FN1, GCP5, VCAN, NCAN, NRP1).[29]
- Clusters 1 and 5 were annotated as "reactive/stressed astrocytes" (1,002 nuclei), since these clusters were almost exclusively seen in MS tissue, and they belonged to the same terminal branch of the cluster tree map. They enriched for unfolded protein response (HSP90AA1, SERPINH1, HSPH1, DNAJB1, BAG3, HSPB1, HSPD1, PPP1R15A, HSPA9, HSP90AB1) and regulation of cell death (NOD1, BAG3, ARHGEF3, ACTN1, HSPB1, CD44, HSPD1, BCL6, GADD45A, GRK5, STK40, HSPA9, FGFR1, FOS, HSP90AB1, MTCH1, TRIO, NPC1, STAT3, ANO6, APC, ITGB1, FYN, ACTN2, IRS2, HIF1A, BNIP3L, PRKD1). Cluster 1 alone enriched also for response to oxygen level (HIF1A, BNIP3L, VEGFA, NAMPT, HILPDA, HK2, HSPD1, PLOD2, FAS, PDK3, PGK1) and collagen fibril organization (SERPINH1, PXDN, AEBP1, DDR2, P4HA1). On the other hand, cluster 5 alone enriched for oxidoreductase activity, showing high expression of mitochondrial-related genes involved in the respiratory chain electron transport (MT-CO2, MT-CO3, MT-ND4, MT-ND2, MT-ND3, MT-ND1, MT-CYB, MT-CO1, MT-ND5, PLOD2, DHFR). These cells also upregulated LINGO1, a known negative regulator of myelination.
- Cluster 4, annotated as "senescent astrocytes," was a small cluster of 824 nuclei, especially frequent in the core of the MS lesion. Enrichment terms referred to microtubule assembly and microtubule-dependent movement (DNAH9, SPAG17, DNAH11, CFAP43, DNAH6, SPAG16, ZBBX, CFAP54, ULK4, DNAH12, DNAH7, HYDIN, CFAP44, AGBL4, DNAH5, CFAP157, DNAI1, CFAP46, ARMC4, DNAAF1, CFAP61, DNAH3, CFAP221, DNAH10, WDR78, LRGUK, WDR63, RSPH1, TEKT1, CEP126, ROPN1L, LRRC6, CFAP73). Of note, the senescent marker CDKN2A was upregulated in this cluster (Z-score 2.2 vs. nonreactive astrocytes ranging from -0.3 and -0.8). On immunostaining, within the lesion core, we

found both senescent astrocytes ($p16^{INK4}$ GFAP double positive) and microglia ($p16^{INK4}$ IBA1 double positive) (Extended Fig. 7c–d).

- Cluster 6 (590 nuclei) was annotated as "astrocytes inflamed in MS" (AIMS) because of the high differential expression of genes such as GFAP, APOE, VIM, S100B, SOD1, and ferritin (FTL, FTH1). Among the upregulated genes in this cluster were proteins involved in MHC protein complex binding (CD81, B2M). These cells also enriched for response to lipid (CLU, VIM, CST3, S100B, CRYAB, SPARC, AGT, CALM1, GJA1, JUND, ZFP36L2, FAM107A, AQP1, ID3, DDIT4, IGFBP7, TXNIP, SOX9, ANXA1, JUN), response to corticosteroids (S100B, SPARC, CALM1, ZFP36L2, FAM107A, AQP1, DDIT4, IGFBP7, ANXA1), and response to wounding (CST3, GFAP, APOE, ACTG1, ACTB, CD81, SPARC, H3F3B, CSRP1, GJA1, PKM, ZFP36L2, DDR1, SOD1, ID3, ANXA1, JUN). Astrocytic dysregulation of cholesterol synthesis (upregulation of APOE and downregulation of ABCA1 in AIMS vs. nonreactive astrocytes) has been implicated in limiting cholesterol availability to oligodendrocytes and neurons for remyelination and synaptic plasticity, respectively.[30] Interestingly, previously described proinflammatory ("A1") genes[31] (C3, SERPING1, AMIGO2, SRGN, PSMB8, FBLN5) showed potentially higher expression in AIMS (Z-scores between 1.3 and 2.6 relative to other astrocyte clusters), though this did not reach our statistical significance criteria (adjusted $p<0.05$, log fold-change threshold $>0.25$, and presence in ≥25% of nuclei in the cluster). GBP2, another A1 marker, was significantly upregulated in one of the other reactive populations (cluster 1). We also specifically looked for the relative expression of complement genes among astrocyte clusters. C3 and C1Q(A,B,C) had, respectively, on average 8.9 and 20-fold increased expression in AIMS compared to nonreactive astrocytes, supporting their cooperative role in antibody-triggered, microglia-mediated tissue damage, and, potentially (considering that axons traversing chronic active lesions eventually reach grey matter) synapse remodeling.[32,33] When compared across pathological lesion stages, the AIMS cluster was especially frequent at the chronic active lesion edge (11% of all astrocytes). At tissue validation, we identified AIMS at the chronic active edge using GFAP, vimentin (VIM), and APOE staining in cells negative for IBA1 or CD68 (Fig. 2h as well as Extended Fig. 4, insets 9–10). Collaterally, in tissue, we found that AQP1 (upregulated in this cluster) is specific to white matter, but not grey matter, astrocytes.
- Cluster 7 was annotated as "perinodal astrocytes" (469 nuclei) for the presence of genes related to the axon-myelin interaction (IL1RAPL1, KIRREL3, UNC5C, ANK3, CNTN2, MAG, LRP2, MYO1D, MBP, SHTN1, BIN1, DNM3, GRM3, APBB2, AGTPBP1, SPOCK1, MAP7, ALCAM, DLG1).[18] These astrocytes were frequently seen in white matter of healthy controls (11% of all astrocytes) and were slightly reduced in MS tissue (on average 6% of all astrocytes, but <1% in the core of the lesion). For these reasons, they are likely to represent a nonreactive astrocytic population.

Vascular cells: annotation and pathway analysis

We analysed clusters initially annotated as vascular cells (1,708 nuclei in clusters 11 and 13 of the initial UMAP). Cluster 11 (955 nuclei) was identified as endothelial cells based on the markers CLDN5 and PECAM1, whereas cluster 13 (753 nuclei) was a mix of pericytes and vascular smooth muscle cells based on the markers PDGFRB and VIM and myosin-related genes such as MYLK. In MS vs. healthy brain tissue, enrichment terms for endothelial cells referred to unfolded protein response (HSP90AB1, HSP90AA1,

PTGES3, HSPA1A, DNAJB1, HSPD1, DNAJA1, HSPA8) and VEGFA-VEGFR2 signalling pathways (FLT1, HSP90AA1, ADAMTS9, HSPA1A, STAT3, DNAJA1, MEF2C, RAP1B, ERG, CGNL1). For pericytes/vascular smooth muscle cells, there were only 6 differentially expressed genes, implicating IL4 and IL13-signaling (RORA, ZEB1, HSP90AA1) and adherens junctions (PARD3, SORBS1).

Our subclustering analysis of vascular cells identified 8 transcriptionally distinct vascular populations. The top 100 differentially expressed genes for each cluster are listed in Supplementary Table 13. Hereafter the detailed annotation and description of all clusters:

- Clusters 1, 2, 4, and 7 were annotated as endothelial cells because of the high differential expression of genes such as CLDN5. Enrichment terms for cluster 1 referred to transport across the blood-brain barrier (ABCB1, MFSD2A, SLC1A1, ABCG2), anchoring junction (FLT1, PODXL, ITGA6, CLDN5, PPFIBP1, CGNL1, RAPGEF2, ARHGAP31, EPHA4, BSG, NEDD9, YES1, MYO1E, ITGA1, AFDN, HMCN1), and transport of small molecules (SLC7A5, ABCB1, ATP10A, SLC7A1, SLCO4A1, SLC16A1, SLC39A10, ANO2, SLC1A1, SLC39A8, ABCG2, ATP7B, SLCO2B1, BSG, A2M, MLKL). For cluster 2, enrichment terms included structural constituent of ribosome (RPL3, RPL13, RPS23, RPS27A, RPL10, RPL15, RPL23A, RPS6, RPS3, RPL30, RPLP1, RPL41, RPL29, RPS2, RPL19, RPL28, RPL8, RPS19, RPL14, RPSA, RPS9, RPL11, RPL6, RPS15), MHC class I protein complex (B2M, HLA-E, HLA-B, HLA-C, HLA-A), and interferon gamma signalling (B2M, MT2A, HLA-E, HLA-B, HLA-C, HLA-A). Cluster 4 enriched for blood vessel development (RAMP3, CEMIP2, PIK3C2A, SLC1A1, C1GALT1, IL6R, THSD7A, BMPR2, SMAD1, CALCRL, SEMA6A, ADAMTS9, CDH2, TEK, NRP1, HIF1A) and cluster 7 (only 72 nuclei) for cell differentiation and cell junction.
- Cluster 3 was annotated as pericytes (PDGFRB). Enrichment terms referred to signalling and ion transmembrane transport activity (SLC38A11, SLC30A10, TRPC3, GJC1, SLC6A12, SLC19A1, ABCC9, ATP1A2, CACNA1C, SLC6A1, TRPC4, SLC12A7, GRID2, SLC6A13, SLC12A2, KCNE4, GRID1, ATP1B3).
- Cluster 5 was annotated as vascular smooth muscle cells because of the high differential expression of genes involved in smooth muscle contraction, such as myosin-related genes (MYLK, MYL9, MYOCD, MYH11), and actin-related genes (ACTA2). Enrichment terms referred to muscle contraction (MYH11, MYOCD, MYL9, PRKG1, CALD1, CACNA1C, CTNNA3, FLNA, ACTA2, PLCE1, MYLK, CRYAB, CACNB2, DMD, PDE5A) and circulatory system development (MYOCD, NOTCH3, MCAM, APOE, SMOC2, ACTA2, PDGFRB, COL4A1, COL4A2, LTBP1, MFGE8, MYLK, NR2F2, JAG1, HIPK2, ADAMTS1, HSPB1, NR4A1, RHOB).
- For cluster 0, a clear annotation was elusive. No expression of CLDN5 was seen, and only 25% of nuclei expressed the pericyte marker PDGFRB. Interestingly, we found high expression of C1R and C1S (part of the complement C1 complex). Enrichment terms included extracellular matrix structural constituent (FBLN1, LAMA2, DCN, FBN1, COL1A2, LAMB1, COL12A1, COL15A1, FBLN5, THBS2, SRPX2, COL6A3, LTBP2, PRELP, ELN, COL11A1, LAMA4), blood vessel development (DCN, PRICKLE1, RORA, COL1A2, CYP1B1, LRP1, COL15A1, FBLN5, THBS2, F3, SRPX2, TGFBR3, ALDH1A2, PRKCA, PRRX1, APOD, LAMA4, PTN, RUNX1, SGCD, PCSK5), and heparin binding (NAV2, FBN1, THBS2, TGFBR3, LTBP2, PRELP, COL11A1, PTN).

- Cluster 6 was a small cluster of 94 nuclei possibly representing misclassified oligodendrocytes cells (MOBP, PLP1).

The low number of vascular cells (with only 65 nuclei belonging to healthy control brain tissue) prevents reliable identification and interpretation of differences across pathological conditions. Overall, in MS tissue compared to healthy brain tissue, we found a proportionally higher number of endothelial cells involved in transport across the blood-brain barrier (cluster 1) and blood vessel development (cluster 4), potentially suggesting increased trafficking across the blood-brain barrier and neovascularization, respectively. No significant differences were seen for pericytes and vascular smooth muscle cells.

Drug-target interaction *in silico* analysis of MIMS

The centrality of MIMS shown by the glial interactome analysis prompted us to predict, based on their transcriptome profile, the effects of known pharmacological compounds. A complete list of compounds is provided in Supplementary Tables 10 and 11. Interestingly, among the predicted compounds, sex hormones showed complex effects on MIMS, with androstanolone as an overall activator (activation of LPL, FTL, FTH1, APOE, LRP1, TMSB4X, ACSL1 and inhibition of COX3, ND3) and estradiol as an overall inhibitor (inhibition of LPL, PTPRG, IL6 and activation of APOE, JUNB), especially in MIMS-foamy. Other top compounds predicted to inhibit both phenotypes of MIMS are statins (ABCA1, ABCG1, CD63, CYBA), including atorvastatin, fluvastatin, and simvastatin; retinoic acid (retinoid X receptors have been implicated also in OPC differentiation, effective myelin debris clearance, and accelerated remyelination);[34,35] ibudilast (inhibition of PDE3B and PDE8A); and quercetin (a flavonoid implemented in senotherapy protocols, mediated by inhibition of AKR1B1 and SGK1 in MIMS-foamy, and by inhibition of AKR1B1 and TOP2B in MIMS-iron). Of note, ibrutinib, a known Bruton's tyrosine kinase (BTK)-inhibitor affecting B-cell survival, has a predicted inhibitory effect on MIMS-iron only (inhibition of RIPK2, a potent activator of NFKB and inducer of apoptosis, as well as an unspecified effect on TXNRD1, a thioredoxin reductase enzyme of which isoform 5 mediates cell death induced by a combination of IFN-beta and retinoic acid). BTK is also a key mediator in the signalling cascade downstream of both the B-cell receptor and the Fc-ɣ receptor,[36] both of which are likely to be involved in the lymphocyte-microglia axis found here to be at the centre of chronic active MS lesion pathophysiology. Some of the aforementioned compounds have been already tested in MS in phase 2 clinical trials, including BTK inhibitors (evobrutinib,[37] tolebrutinib), ibudilast,[38] estriol,[39] and simvastatin,[40] but since reliable in vivo imaging of chronic active lesions has only recently become possible,[41,42] there are no clinical or radiological data regarding the response of those lesions to these previously trialled drugs.

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