Parenchymal border macrophages regulate the flow dynamics of the cerebrospinal fluid

Macrophages are important players in the maintenance of tissue homeostasis. Perivascular and leptomeningeal macrophages reside near the central nervous system (CNS) parenchyma, and their role in CNS physiology has not been sufficiently well studied. Given their continuous interaction with the cerebrospinal fluid (CSF) and strategic positioning, we refer to these cells collectively as parenchymal border macrophages (PBMs). Here we demonstrate that PBMs regulate CSF flow dynamics.

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We identify a subpopulation of PBMs that express high levels of CD163 and LYVE1 (scavenger receptor proteins), closely associated with the brain arterial tree, and show that LYVE1+ PBMs regulate arterial motion that drives CSF flow. Pharmacological or genetic depletion of PBMs led to accumulation of extracellular matrix proteins, obstructing CSF access to perivascular spaces and impairing CNS perfusion and clearance. Ageing-associated alterations in PBMs and impairment of CSF dynamics were restored after intracisternal injection of macrophage colony-stimulating factor.

The CNS myeloid niche in the homeostatic brain comprises microglia and perivascular macrophages. Unlike microglia, which are located within the brain parenchyma, PBMs reside in the leptomeninges and perivascular spaces along the vasculature, and are therefore constantly in direct contact with the CSF. Previous studies have suggested a detrimental role for such macrophages in chronic hypertension, AD, stroke and experimental autoimmune encephalomyelitis. The functions of PBMs in brain homeostasis, however, are still largely unexplored.

Here we show that PBMs regulate CSF flow dynamics, an integral aspect of brain physiology, under homeostatic conditions. We identify arterial-associated PBMs that display a transcriptomic profile of scavenger cells and control extracellular matrix (ECM) remodelling, which affects arterial motion, a driving force of CSF flow dynamics. Depletion of PBMs results in the accumulation of ECM proteins and impairment of brain perfusion by the CSF. We demonstrate that normal ageing is associated with PBM dysfunction. Moreover, treatment of aged mice with macrophage colony-stimulating factor (M-CSF) improves CSF dynamics.

The meninges, a tripartite membranous covering of the brain parenchyma, are densely populated by immune cells and their derived cytokines can affect mouse behaviour. Cytokines from the periphery and the meninges can be carried along in CSF, which is driven by arterial pulsation and vasomotion. The removal and subsequent clearance of brain metabolites may be facilitated through perivascular lymphatics, the removal and subsequent clearance of brain metabolites. Antigens are transported along the same routes into the dura, where they are sampled by dural antigen-presenting cells and presented to patrolling T cells to ensure immune surveillance of the CNS. Some of these brain-derived antigens subsequently also drain into the deep cervical lymph nodes (dCLNs) through meningeal lymphatic vessels.

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The CNS myeloid niche in the homeostatic brain comprises microglia and perivascular macrophages (we collectively term these two populations of border macrophages as PBMs). Unlike microglia, which are located within the brain parenchyma, PBMs reside in the leptomeninges and perivascular spaces along the vasculature, and are therefore constantly in direct contact with the CSF. Previous studies have suggested a detrimental role for such macrophages in chronic hypertension, AD, stroke and experimental autoimmune encephalomyelitis. The functions of PBMs in brain homeostasis, however, are still largely unexplored.

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PBMss sample CSF and regulate its flow dynamics

PBMs are found in leptomeningeal and perivascular spaces in the brain, at the vicinity of larger blood vessels, and can be distinguished from microglia by their location and expression of the mannose receptor CD206 (ref. 16) (Fig. 1a, Extended Data Fig. 1a–c and Supplementary Video 1). We were able to distinguish two subtypes of PBMs on the basis of their expression of either LYVE1 or major histocompatibility complex II (MHCII) (Fig. 1b,c). Using flow cytometry, PBMs were distinguished using MHCII and CD38 as a substitute to LYVE1, as previously described11 (Extended Data Fig. 1d).

Perivascular spaces are filled with CSF and constitute an interface between blood and the CNS parenchyma12. CSF flows along the perivascular space, can cross astrocytic endfeet and flow into the brain (a process termed glymphatic)9,23. We studied CSF flow dynamics by injecting fluorescent tracers into the mouse cisterna magna and then assessing tracer diffusion into the brain. Fluorescent ovalbumin (OVA) was delivered through intra-cisterna magna (i.c.m.) injection, and the mice were perfused after 1 h. The entire brain was extracted, fixed and imaged by light sheet microscopy (Extended Data Fig. 1e and Supplementary Video 2) or by stereomicroscopy (Fig. 1d and Extended Data Fig. 1f). Using these methods, we observed that OVA was mostly located in the regions of the olfactory bulbs, the cerebellum and the middle cerebral artery (MCA) (Fig. 1d).

Although most of the tracer accumulated at the perivascular space, 28.9% of tracer was sampled by cells (Fig. 1d and Extended Data Fig. 1f).

Tracer penetration into the brain parenchyma is reportedly greater for small fluorescent tracers13. However, we found that tracers, independent of their size, accumulated in both perivascular and leptomeningeal spaces in CD206+ macrophages (or PBMs; Fig. 1f and Extended Data Fig. 1g). We also observed tracer uptake by PBMs when tracers were infused into the striatum (Extended Data Fig. 1h), which suggested that PBMs sample CSF content on its way into and out of the brain. Indeed, we observed double-positive PBMs 2 h after the tracers were co-injected into both the striatum and CSF, although CSF influx was reduced owing to concomitant intra-striatal injection, as previously described13 (Extended Data Fig. 1i).

Given the close association of PBMs with CSF, we hypothesized that CSF flow dynamics may be partially controlled by PBMs. To test this hypothesis, liposomes containing clodronate (CLO) were administered through i.c.m. injections to deplete PBMs (about 75% depletion was achieved when brain tissue was examined 1 week later; Fig. 1g–i). We confirmed that both the number and the morphology of microglial cells were not affected by i.c.m. CLO liposome administration (Extended Data Fig. 2a). Immunohistochemistry (IHC) and single-cell RNA-sequencing (scRNA-seq) analyses also demonstrated that PBMs are the major population of brain border-associated cells that phagocytosed i.c.m.-injected Dil-labelled liposomes. By contrast, microglia and other stromal cells did not sample the tracer (Extended Data Fig. 2b–e) and hence could not be directly affected by the liposomes. Notably, we observed two major PBM subtypes on the basis of their gene expression.

**Fig. 1** | PBMs sample CSF and regulate its flow dynamics. a, CD206+ PBMs are located in close association with blood vessels stained positive by intravenous injection of lectin. b, CD206+ PBMs can be separated into two major subtypes by their expression of LYVE1 or MHCII co-stained for DAPI. c, Quantification of LYVE1 versus MHCII+ PBM subtypes (average of both perivascular space and leptomeninges). n = 5 mice. d, WT mice received an i.c.m. injection of Alexa 647-conjugated OVA. Mice were perfused 1 h after OVA injection. Representative stereomicroscopy images showing whole brain OVA distribution from top (top row) and side (bottom row) views. OVA is mostly distributed around the olfactory bulbs (Ob), MCA and cerebellum (Cblm). e, OVA distribution in brain coronal sections (co-stained for DAPI) is largely found in leptomeninges and penetrating blood vessels. At higher magnification, OVA distribution also appears to be cellular (inset). f, OVA+ cells express the PBM marker CD206. g, Schematic of experiment. One week after PBM depletion using i.c.m.-injected CLO liposomes, mice received an i.c.m. injection of OVA and perfused 1 h later. h, Representative images of brain sections showing CD206 and intravenous-injected lectin staining. i, Quantification of CD206+ cells. n = 5 mice per group; two-tailed unpaired Welch’s t-test. j, Representative images showing OVA distribution in whole brains in PBM-depleted and control mice (left) and corresponding quantification (right). For j and k, n = 6 mice per group; two-tailed unpaired Welch’s t-test. All data are presented as the mean ± s.e.m. Scale bars, 50 μm (b), 100 μm (f), 200 μm (e, inset), 500 μm (a), 1 mm (d, inset), 2 mm (e,k) or 5 mm (d,j). The illustration of the mouse in g is from Servier Medical Art, CC BY 3.0. 

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of Lyve1 and MHCII, as well as by staining and flow cytometry (Extended Data Fig. 2f–i). Specifically, CD206+Lyve1+MHCII+PBMs were highly phagocytic and endocytic cells, expressing high levels of scavenger receptors such as Cd163, Mcrl, Lyve1, Mrsl and Siglec1. Notably, these cells also upregulated genes involved in the interferon-γ (IFNy) pathway such as Ifrg8, Ifitm2 and Ifitm6 (Extended Data Fig. 2h,i). Gene ontology pathway analysis highlighted important roles of Lyve1+ PBMs in metabolic processes and chemotaxis (Extended Data Fig. 2l). Conversely, CD206+Lyve1–MHCII+PBMs upregulated pathways that are involved in immune response, response to viruses, cytokine production, cell–cell adhesion and antigen presentation (Extended Data Fig. 2h,i). These results indicated that the two PBM subtypes have different functions.

To assess the role of PBMs in CSF dynamics, fluorescent OVA was given through i.c.m. injection 1 week after PBM depletion. After allowing the tracer to diffuse freely for 1 h, mice were perfused and the whole brain was extracted, fixed and imaged by stereomicroscopy (Fig. 1f). In PBM-depleted mice, the OVA coverage was significantly reduced (Fig. 1f). Brains were then sectioned to evaluate OVA coverage in coronal sections, a method commonly used to evaluate CSF influx23 (Fig. 1k). OVA coverage of brain slices was also significantly reduced in PBM-depleted mice (Fig. 1k). Three days after CLO treatment, CSF flow was impaired to a lesser extent than 1 week after, which correlates with a lower level of (nevertheless, significant) PBM depletion at this time point (Extended Data Fig. 2j–m).

Our group recently showed that CSF flow is impaired after dural lymphatic ablation23. After 3 days of CLO treatment, the number of CD206+ dural macrophages located at the vicinity of the superior sagittal sinus was reduced (Extended Data Fig. 2n–o). Conversely, there was no effect of CLO treatment after 1 week on either dural lymphatic vessels or dural CD206+ macrophages, which indicates that the observed impairment of CSF flow in this study cannot be attributed to dural lymphatic ablation (Extended Data Fig. 2p–s). There was also no effect of CLO treatment on choroid plexus CD206+ macrophages (Extended Data Fig. 2t). However, a population of Iba1+ cells were absent in dCLNs after CLO treatment (Extended Data Fig. 2u), which are presumably sinus subcapsular macrophages that were depleted once CLO liposomes drained to dCLNs.

We repeated the CSF flow experiment using 4 kDa dextran conjugated with fluorescein isothiocyanate (FITC–dextran) or 3 kDa Texas Red, which are more diffusive owing to their small molecular weight. Similar to OVA, the influx of small tracers was impaired after PBM depletion (Extended Data Fig. 3a,b). Furthermore, we observed significant accumulation of tracers (both OVA and FITC–dextran) in the brain parenchyma 1 h after intra-striatal injection in PBM-depleted mice. This result suggests that both influx and efflux of CSF are impaired after PBM depletion (Extended Data Fig. 3c,d) or that CSF influx and efflux are interdependent, as previously described (for example, intra-striatal injection impairs influx of CSF tracers29). To assess whether the impairment in CSF flow dynamics affects CSF protein content itself, we performed a proteomics analysis of CSF sampled from PBM-depleted mice and control mice (Extended Data Fig. 3e). Accumulation of synapse-related proteins in PBM-depleted mice, such as NRXN1, PTPRS, NRCAM and CDH2, was observed (Extended Data Fig. 3f–h). Moreover, there was accumulation of clusterin (CLU), apolipoprotein E (APOE) and amyloid precursor protein (APP), proteins that have been associated with AD, in the CSF of PBM-depleted mice (Extended Data Fig. 3i–k).

Next we used magnetic resonance imaging (MRI) to better evaluate the dynamics of CSF flow in vivo20,37 (Fig. 2a). Diffusion of the contrast agent (Dotarem; 0.754 kDa) over time was reduced in PBM-depleted compared with control-treated mice (Fig. 2b,c and Supplementary Videos 3 and 4), without any notable effect of PBM depletion on ventricular size (Extended Data Fig. 3l–n). Intracranial pressure was also increased 1 week after PBM depletion, which normalized 3 weeks after depletion (Extended Data Fig. 3o), probably due to cells starting to repopulate the niche27,28. MRI analyses also revealed a reduction of tracer diffusion at the vicinity of the MCA (Extended Data Fig. 3p–r). IHC analyses showed that OVA coverage of dCLNs was reduced after PBM depletion (Extended Data Fig. 3s–v). However, there was no difference in OVA drainage to dCLNs, as assessed by in vivo imaging (Extended Data Fig. 3w–y). This is probably because of the rapid efflux of OVA from the dCLNs in PBM-depleted mice, as evidenced from live imaging of drainage (Extended Data Fig. 3z). This in turn is probably as a result of sinus subcapsular macrophage depletion by drained CLO liposomes (Extended Data Fig. 2u).

To better understand how PBM depletion affects CSF flow dynamics, we developed a new in vivo approach that enabled us to monitor fluorescent tracer movement over time. CSF tracers rapidly diffuse at the proximal part of the MCA perivascular space23,39. In addition, given the observed reduced tracer coverage around the MCA by MRI (Extended Data Fig. 3p–r), we decided to visualize fluorescent macromolecule movement through the intact lateral parietal bone after retraction of the right temporo-parietal muscle (Fig. 2d,e). Immediately after i.c.m. injection of fluorescent OVA, mice were turned onto their side for in vivo stereomicroscopy imaging of the proximal part of the MCA through the intact skull (Fig. 2d and Extended Data Fig. 4a–c). Using this method, we observed in vivo that OVA rapidly localized at the MCA perivascular space (Extended Data Fig. 4d) and was sampled by perivascular cells (Extended Data Fig. 4e). We validated this method by exposing mice to different anesthetics that either enhance (ketamine and xylazine (KX) cocktail) or inhibit (isoflurane) the movement of CSF tracers20,30 (Extended Data Fig. 4f–j). Using this approach, we observed that OVA coverage over time was strongly reduced in PBM-depleted mice at the MCA level. This was in accordance with ex vivo results showing that global tracer coverage was reduced (Fig. 2f,g and Supplementary Videos 5 and 6).

Collectively, these data provide evidence that PBMs are strategically located at the interface between blood and the brain parenchyma, they sample CSF and regulate its flow dynamics.

**PBM extracellular matrix and arterial motion**

After PBM depletion, OVA was barely able to reach the perivascular space of penetrating vessels (Extended Data Fig. 4k). This raised questions about the morphology of the perivascular space after PBM depletion. Aquaporin-4 (AQ4), a water channel present in the astrocytic endfeet that form the glia limitans (that is, the outer layer of the perivascular space), has been proposed as a mediator of CSF influx23. Using IHC, we were unable to detect any effect of PBM depletion on AQ4 coverage or AQ4 polarization (Extended Data Fig. 4l–n). The space between the two markers (arguably representing the perivascular space) was substantially smaller in PBM-depleted mice than in control mice (Extended Data Fig. 4o–q). Using i.c.m.-injected fluorescent microbeads4, we were able to assess perivascular space in vivo (Extended Data Fig. 4r–t). However, this method did not allow assessment of the perivascular space in PBM-depleted mice because few beads could be detected (Extended Data Fig. 4s,t). To circumvent in vivo imaging, we perfused the mice with PBS without paraformaldehyde (PFA) to preserve the perivascular space, and bead accumulation was assessed at the vicinity of the MCA4 (Extended Data Fig. 4u,v). First, we compared perivascular space measurements in PBS-treated mice using both methods. A slight decrease in ex vivo compared to in vivo measurements was observed (Extended Data Fig. 4u–w). Using this method, the bead coverage was strongly reduced in PBM-depleted mice (Extended Data Fig. 4x). Although the perivascular space and MCA diameter did not change (Extended Data Fig. 4y), the space filled by the beads was reduced in PBM-depleted mice (Extended Data Fig. 4z).

The changes in CSF flow dynamics were accompanied by mild behavioural alterations (Extended Data Fig. 5). PBM-depleted mice froze more on the first day of the cued-fear conditioning test (Extended Data Fig. 5a).
To better understand the biology of PBMs, we performed scRNA-seq Data Fig. 5a). However, PBM-depleted mice did not show any deficits in the following parameters: anxiety (assessed using the elevated plus maze test; Extended Data Fig. 5b); general motor activity (assessed using the open-field test; Extended Data Fig. 5c); response to stress (assessed using the forced-swim test; Extended Data Fig. 5d); or social recognition (assessed using the three-chamber test; Extended Data Fig. 5e). We also measured vital signs such as respiratory rate, heart rate and arterial pulsations and diameter, and did not find any difference between groups (Extended Data Fig. 5f).

To understand whether stromal cells (that is, endothelial cells and mural cells such as pericytes, vascular smooth muscle cells (VSMCs) and fibroblasts) are affected by PBM depletion, we sorted CD45–CD13+CD31– mural cells such as pericytes, vascular smooth muscle cells (VSMCs) and fibroblasts (assessed using the forced-swim test; Extended Data Fig. 5d); or social recognition (assessed using the three-chamber test; Extended Data Fig. 5e). We also measured vital signs such as respiratory rate, heart rate and arterial pulsations and diameter, and did not find any difference between groups (Extended Data Fig. 5f).

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PBMs regulate CSF flow dynamics through ECM remodelling and arterial motion. a, Left, representative images of brain coronal sections showing collagen IV and DAPI staining. Right, high magnification images showing collagen IV association with CD31+ blood vessels. b, Quantification of collagen IV coverage. c, Quantification of laminin coverage. d, Quantification of MMP activity (fluorescence spectrometry). e, Quantification of MMP activity. n = 6 mice per group. MFI, mean fluorescence intensity. f, Left, schematic of experiment. One week after CLO or PBS treatment, mice were imaged using photoacoustic microscopy. Right, representative images showing vessels before (left) and during (middle) 10% CO2 challenge and corresponding kymographs (right). g, Quantification of vessel diameter fold-increase over time. n = 7 mice (treated with PBS) or 9 mice (treated with CLO). h, Quantification of arterial vessel diameter fold-increase during CO2 challenge. n = 7 mice (treated with PBS) or 9 mice (treated with CLO).

including Mrc1 and Ms4a7) and obtained 5 PBM clusters (Fig. 3k). To delineate crosstalk between PBMs and other cell types, we used the RNA Magnet algorithm, which predicts paired physical and signalling interactions between cell types33. Notably, we found that cluster 2, which exhibited high expression of scavenger markers such as Lyve1 and Cd163, interacted specifically with VSMCs, which are located at the arterial level (Fig. 3l). Spatial proximity between PBMs and VSMCs was confirmed by electron microscopy, which indicated that these two cell types may interact, although they are separated by the basal lamina (Extended Data Fig. 7b). These results confirmed recent findings that described important interactions between VSMCs and perivascular macrophages to allow their migration at the perivascular space.1. Looking at differentially expressed genes between PBM clusters, cluster 2 was identified as potential professional scavenger cells (Cd163, Cd38, Lyve1, Msr1 and Cd36) when compared to the other clusters, which expressed genes related to antigen presentation (H2-Ab1, Cd74, Cd33, Cd14 and Nlrp3) (Extended Data Fig. 7c–e). Immunostaining confirmed that LYVE1+ PBMs were highly concentrated at the vicinity of αSMA+ brain arteries and arterioles, whereas MHCII+ PBMs were mostly localized to αSMA+ brain blood vessels (Extended Data Fig. 7f,g). We then took advantage of LYVE1 expression by PBMs to genetically target these cells using Lyve1Cre:Csfr1fl/fl mice (Cre+; Extended Data Fig. 7h). Mice at 3 months of age showed about 50% depletion of PBMs compared with their littermate controls not expressing Cre (Cre−; Extended Data Fig. 7j). We confirmed by flow cytometry that CD38+ PBMs were depleted by this genetic ablation, without affecting microglial cells or MHCII+ PBMs (Extended Data Fig. 7j–n). In support of the results from pharmacological ablation experiments, genetic ablation of LYVE1+ PBMs also affected ECM protein levels (Extended Data Fig. 7o,p) and CSF influx (Extended Data Fig. 7q–v). However, there were no changes in vessel coverage, MMP activity or intracranial pressure (Extended Data Fig. 7w–y), and CSF flow impairment was more subtle in these mice (Extended Data Fig. 7q–v). The differences may be because these mice have reduced PBM depletion compared with mice treated with CLO, or because a lifelong reduction in CSF flow resulted in adaptation or emergence of alternative pathways to regulate intracranial pressure.

In summary, these observations suggest that PBMs regulate CSF dynamics along the perivascular space and its efflux and influx of the brain parenchyma. The mechanism underlying PBM regulation of CSF dynamics is based on their ability to regulate ECM remodelling, which in turn affects arterial stiffness.

PBMs in ageing and AD

CSF flow is impaired in old mice, and this impairment could be ameliorated in part through enhancement of meningeal lymphatic vessels.32.
We proposed that PBMs participate in the age-related deterioration of CSF dynamics. MRI analyses demonstrated that CSF flow is globally impaired in old mice. Fluorescent tracers also confirmed impairment in brain coronal sections (Fig. 4a–c and Extended Data Fig. 8a–f). Comparison of the PBMs in young adult (3-month-old) and old (24-month-old) mice showed no difference in overall CD206+ cell numbers, but aged mice exhibited a significant reduction in LYVE1+ and an increase in MHCII+ cells, which is in agreement with previous reports (Fig. 4d–g and Extended Data Fig. 8g–k). CD38+ PBMs in young mice were the major cell type that phagocytosed i.c.m. injected pHrodo particles (Extended Data Fig. 8l–o). The change in PBM phenotype observed in aged mice appeared to be associated with impaired pHrodo particle phagocytosis (Extended Data Fig. 8p–r), reduced functional perivascular space filled by the beads (Extended Data Fig. 8s–y) and accumulation of ECM proteins (Fig. 4h–j and Extended Data Fig. 8z).

M-CSF has been previously shown to improve pathophysiology of AD, presumably through the enhancement of amyloid-β (Aβ) phagocytosis by blood-derived monocytes. We proposed that such acute activation of PBMs in old mice might enhance their ECM degradation.
Fig. 5 | PBMs in human AD and a S×FAD mouse model of amyloidosis.

a, Schematic of experiment. S×FAD mice received an i.c.m. injection of PBS or CLO liposomes. One month later, mice received an i.c.m. injection of OVA and brains were collected 1h later. b, Representative brain coronal section images showing Aβ plaque load (left) and corresponding quantification (right). Scale bar, 2 mm. n = 7 mice (treated with PBS) or 8 mice (treated with CLO); two-tailed unpaired Welch’s t-test. c, Schematic of experiment. scRNA-seq of the brain cortex from 6–7-month-old S×FAD mice and their WT littermates. n = 8 mice per group. d, Macrophage populations were isolated from the S×FAD mice and subjected to scRNA-seq. Nine clusters were identified. e, Volcano plot corresponding to downregulated and upregulated genes in PBMs comparing S×FAD mice and their WT littermates. F-test with adjusted degrees of freedom based on weights calculated per gene with a zero-inflation model and Benjamini–Hochberg adjusted P values. f, Human single-nucleus RNA-seq from patients with familial AD and from unaffected individuals. Six cell populations were used in this dataset: microglia, fibroblasts, endothelial cells (ECs), mural cells, lymphocytes and PBMs. g, Volcano plot corresponding to downregulated and upregulated genes in PBMs comparing patients with familial AD and unaffected individuals. F-test with adjusted degrees of freedom based on weights calculated per gene with a zero-inflation model and Benjamini–Hochberg adjusted P values. h, Violin plots corresponding to CD163 gene expression from both human single-nucleus RNA-seq (left) and mouse scRNA-seq (right). All data are presented as the mean ± s.e.m. The illustrations of mice in a and c are from Servier Medical Art, CC BY 3.0.

Our scRNA-seq results revealed that VSMC-associated PBMs can be differentiated from other PBMs by their expression of scavenger receptors (Extended Data Fig. 7e–j). Pathway analysis confirmed that cluster 2 showed upregulated genes characteristic of receptor-mediated endocytosis and phagocytosis (Extended Data Fig. 7e). This analysis also showed that cluster 2 was linked to a cellular response to Aβ. Furthermore, it has been shown that depletion of perivascular macrophages worsens outcome in a mouse model of cerebral amyloid angiopathy (CAA)15, which suggests that PBMs are processing brain-derived Aβ. Finally, our CSF proteomics data indicated that PBM depletion leads to an accumulation of AD-associated risk factors such as CLU, APOE and APP (Extended Data Fig. 3i–k). We therefore proposed that PBMs might be involved in Aβ clearance. To test this hypothesis, 2-month-old S×FAD mice (a mouse model of AD) each received a single i.c.m. injection of CLO liposomes or, as a control, PBS liposomes (Fig. 5a). CSF flow was impaired in PBM-depleted S×FAD mice compared with their S×FAD control littermates (Extended Data Fig. 10a,b). When their Aβ plaque loads were evaluated 1 month later, PBM-depleted S×FAD mice exhibited significantly increased plaque load compared with their S×FAD control littermates (Fig. 5b), specifically in the brain cortex and amygdala (Extended Data Fig. 10c). To better understand the role of PBMs in AD pathophysiology, we performed scRNA-seq on brains from 6–7-month-old S×FAD and their wild-type (WT) littermates (Fig. 5c and Extended Data Fig. 10d). We reclustered all macrophages and identified PBMs using the gene marker Mrc1 (Fig. 5d and Extended Data Fig. 10e). We also identified a damage-associated microglia (DAM) cluster specifically in S×FAD mice, as previously described16 (Extended Data Fig. 10f). PBMs from S×FAD mice exhibited altered phagocytosis and endocytosis and response to IFNγ pathways (Fig. 5e and Extended Data Fig. 10g). We confirmed using the RNA Magnet algorithm that PBMs interact with mural cells, notably VSMCs and fibroblasts (fibroblast-like cells) (Extended Data Fig. 10h). Of note, a human dataset that we had previously used to assess microglial function in AD18 also contained a small population of PBMs (Fig. 5f). PBM populations from unaffected individuals and from patients with familial AD substantially differed, with 445 upregulated and 249 downregulated genes, respectively (Fig. 5g and Extended Data Fig. 10i). Among the most notably dysregulated gene signatures in human PBMs from patients with familial AD were those involved in phagocytosis and endocytosis (CD163 expression) and IFNγ signalling (Fig. 5h and Extended Data Fig. 10j), which recapitulated the findings from the AD mouse model. Notably, both the IFNγ receptor genes Ifngr1 and Ifngr2 were more highly expressed in brain immune cells (that is, microglia and PBMs) than in stromal cells in our mouse single-cell dataset, which indicated an important interaction between PBMs and IFNγ (Extended Data Fig. 10k). To test the possibility that excess IFNγ in CSF may cause dysfunction in CSF dynamics, we injected (i.c.m.) young adult WT mice with IFNγ. These mice exhibited impaired CSF flow compared with mice injected with PBS (Extended Data Fig. 10l).

Collectively, these findings reveal that PBMs are pivotal players in CSF flow dynamics in ageing and in AD (Extended Data Fig. 10n). Therefore, PBMs should be further explored as potential new therapeutic targets.
for AD and other age-associated diseases characterized by protein aggregation and CSF dysfunction.

Discussion

The results of this study demonstrated that perivascular and leptomeningeal macrophages express similar markers and are located around the CNS parenchyma, constantly interacting with CSF. Given their location, function and marker expression, we suggest referring to them as a single functional population, namely PBMs.

PBMs are composed of two major subtypes: LYVE1+MHCIIlo/neg and LYVE1lo/negMHCII+. Although PBMs and microglia are both derived from early erythromyeloid progenitors in the yolk sac, a recent study suggested that LYVE1+ macrophages predominantly originated from embryonic-derived progenitors and are maintained locally in the peritoneal mesothelium. Our data demonstrated that LYVE1+MHCII+ PBMs regulate arterial motion and ECM remodelling (along large vessels and capillaries). Indeed, depletion or dysfunction of PBMs resulted in impaired arterial motion, accumulation of ECM and impairment of CSF flow. Previous studies that used CLO liposomes to deplete perivascular macrophages did not observe major changes in cerebral blood flow. The apparent discrepancy may be a result of the use of cranial windows and dura mater removal used in those studies, which might have masked the effects we observed here with thinned-skull preparations. Spontaneous low-frequency oscillations of arterioles in brain parenchyma have been proposed as the driving force of CSF and interstitial fluid clearance. Notably, this perivascular clearance was impaired in the context of CAA. Moreover, depletion of perivascular macrophages in CAA exacerbates the disease, and perivascular macrophages can produce excessive reactive oxygen species, which then lead to neurovascular dysfunction in the context of hypertension and AD. Together with our data, it is plausible that PBMs are at the interface between spontaneous arterial oscillations, ECM remodelling, neuronal activity and CSF flow.

Notably, aged mice exhibited an increase in LYVE1lo/negMHCII+ PBMs, increased ECM deposition and impaired CSF dynamics. We showed here that treatment of aged mice with M-CSF increased MMP activity, reduced ECM accumulation and acutely restored impaired CSF flow. PBM depletion also resulted in increased accumulation of parenchymal plaques in the 5×FAD mouse model of amyloidosis. PBMs from patients with familial AD exhibited altered expression profiles compared with individuals without AD, including dysregulated pathways involving phagocytosis and endocytosis and IFNγ signalling on PBMs. Similar pathways were also among the most differentially regulated in PBMs of mice from WT compared with 5×FAD mice. Stimulation of PBMs with familial AD exhibited altered expression profiles compared with WT, with dysregulated pathways involving IFNγ signalling and impaired CSF flow. Previous studies that used CLO liposomes to deplete perivascular macrophages did not observe major changes in cerebral blood flow. The apparent discrepancy may be a result of the use of cranial windows and dura mater removal used in those studies, which might have masked the effects we observed here with thinned-skull preparations. Spontaneous low-frequency oscillations of arterioles in brain parenchyma have been proposed as the driving force of CSF and interstitial fluid clearance. Notably, this perivascular clearance was impaired in the context of CAA. Moreover, depletion of perivascular macrophages in CAA exacerbates the disease, and perivascular macrophages can produce excessive reactive oxygen species, which then lead to neurovascular dysfunction in the context of hypertension and AD. Together with our data, it is plausible that PBMs are at the interface between spontaneous arterial oscillations, ECM remodelling, neuronal activity and CSF flow.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41586-022-05397-3.
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Domestically Inherited Alzheimer Network

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Methods

Mice
Mice were bred in-house or obtained from the Jackson Laboratory (JAX) or provided by the National Institutes of Health/National Institute on Aging (24-month-old mice). Mice were housed in a 12-h light–dark cycle in a temperature-controlled and humidity-controlled environment with water and food provided ad libitum. Mice were housed for at least 1 week at the animal facility before any experimentation. Both males and females were used in this study. The following mouse strains were used: C57BL/6J (WT; JAX 000664), C57BL/6-Tg(UbcGFP; JAX 004353), hemizygous B6SJL-Tg (APPswFlon; PSEN1*M146L*L286V)699Vas/Mmjax (S-FAD mice; JAX 008730), Lyve1cre(B6.Cg-Csf1rtm1.2Jwp/J; JAX 021212) and Aldh1l1Cre/ERT2 (B6; JAX 031008). Sample sizes were chosen on the basis of standard power calculations (with α = 0.05 and power of 0.8) performed for similar experiments that have been previously published. Animals from different litters, but within the same experimental group, were selected to ensure randomization. Mice from the same cage received different treatments (for example, in a cage of five mice, two mice received PBS-loaded liposomes and three mice received CLO-loaded liposomes). Treatment was given in a blind manner and could be identified by a corresponding ear tag (for example, for treatment A, the left ear was tagged whereas for treatment B, the right ear was tagged). All experiments were approved by the Institutional Animal Care and Use Committee of the University of Virginia and the Institutional Animal Care and Use Committee of the Washington University in Saint Louis.

Treatments with i.c.m. injections
Mice were anaesthetized using an intraperitoneal injection of KX cocktail (100 mg kg⁻¹ ketamine and 10 mg kg⁻¹ xylazine) diluted in 0.9% Na (saline) solution. The fur of the neck was shaved and cleaned with 70% iodine. Then, mice were placed in a stereotaxic frame to maintain the head in a fixed position, and an ophthalmic solution was applied to prevent dry eyes. The skin from the neck was longitudinally incised, and muscles were retracted using hooks to expose the cisterna magna. The solutions, diluted in aCSF, were injected using a 33-gauge Hamilton syringe (1–5 μl; 2.5 μl min⁻¹). The syringe was left in place for 1 min after injection to prevent backflow. For survival surgeries, the skin was sutured and mice were kept on a heating pad until fully awake. Mice received a subcutaneous injection of ketoprofen (2.5 mg kg⁻¹) at the end of the surgery.

To deplete PBMs, mice received an i.c.m. injection of CLO-loaded liposomes (5 μl; 5 mg ml⁻¹; Fisher Scientific, CLD-8901). The control group consisted of mice that received an i.c.m. injection of PBS-loaded liposomes. The effect of PBM depletion was mostly studied 1 week after liposome injection to avoid any side effects from an inflammatory reaction due to the depletion. Moreover, at 1 week, we observed strong depletion (80–85% depletion), as previously described. We chose to wait for 1 month instead of 1 week to evaluate the mid-to-long-term effect of PBM depletion on plaque accumulation in the S-FAD mouse model of AD.

To evaluate CSF flow dynamics in different contexts, Alexa 647-conjugated OVA (45 kDa; 5 μl; 1 mg ml⁻¹ diluted in aCSF; Thermo Fisher Scientific, O34784), FITC-dextran (4 kDa; 5 μl; 5 mg ml⁻¹ diluted in aCSF; Sigma-Aldrich, 46944), Texas Red dextran (3 kDa; 5 μl; 1 mg ml⁻¹ diluted in aCSF; Fisher Scientific, D3328), fluorescent beads (0.1 μm thick; 5 μl; 1:5 dilution in aCSF; Life Technologies, F8888) or Dotarem (galodinol-based MRI contrast agent; 0.754 kDa; 5 μl; 0.5 mmol ml⁻¹; Guerbet) were injected through i.c.m. and mice were perfused 1 h later.

To evaluate the role of PBMs on CSF flow in old mice, 24-month-old mice received an i.c.m. injection of 5 μl aCSF or M-CSF (10 μg ml⁻¹ diluted in aCSF; Sigma-Aldrich, M9170). Six hours or 24 h later, mice received an i.c.m. injection of OVA to evaluate CSF flow.

To evaluate the effect of IFNγ on CSF flow, young adult mice (2–3 months old) received an i.c.m. injection of 1 μl aCSF or recombinant IFNγ (20 μg ml⁻¹ diluted in aCSF; Fisher Scientific, 485-MI-100/CF). Three hours later, mice received an i.c.m. injection of 5 μl OVA to evaluate CSF flow.

To evaluate PBM phagocytosis, mice received an i.c.m. injection of pHrodo particles (1 mg ml⁻¹ in aCSF; Deep Red Escherichia coli bioparticles, Life Technologies, P35360). These particles emit 647 nm wavelength fluorescence only after being phagocytosed by cells (pH-dependent). Phagocytic activity was measured by pHrodo coverage by IHC, and pHrodo cells was quantified by flow cytometry.

Intrastralial injections
Anaesthetized mice (through KX cocktail) were shaved on the top of the head and placed in a stereotaxic frame. After skin incision, a small cranectomy was made using a drill, and the different solutions were injected using a glass capillary (1 μl; 0.2 μl min⁻¹) (coordinates from the Bregma: anterior–posterior: +1.5 mm; medial–lateral: –1.5 mm; dorsal–ventral: +2.5 mm). The glass capillary was left in place for five additional minutes to prevent backflow. Mice were then sutured and placed on a heating pad until further experiments.

Dobutamine injection
For one experiment, mice received an intraperitoneal injection of dobutamine (40 μg kg⁻¹ diluted in saline; Sigma Aldrich, D6766), a β-adrenergic agonist, or saline as a control, before CSF flow evaluation.

Proteomics analysis of CSF
CSF collection. Mice were anaesthetized using a KX cocktail. The fur of the neck was shaved and cleaned with 70% iodine. Mice were then placed in a stereotaxic frame to maintain the head in a fixed position, and an ophthalmic solution was applied to prevent dry eyes. The skin from the neck was longitudinally incised and muscles were retracted using hooks to expose the cisterna magna. A glass capillary was inserted into the cisterna magna to collect CSF, which was transferred into 1.5 ml Eppendorf tubes for further analyses.

Peptide preparation. CSF (7–10 μl) samples from mice were dried in a speed-vac and solubilized with 30 μl of SDS buffer (4% w/v), 100 mM Tris-HCl pH 8.0, and 0.2% dichloroacetone (DCA)). The protein disulfide bonds were reduced using 100 mM dithiothreitol with heating to 95°C for 10 min. Peptides were prepared as previously described using a modification of the filter-aided sample preparation method. The samples were mixed with 200 μl of 100 mM Tris-HCl buffer, pH 8.5, containing 8 M urea and 0.2% DCA (UA buffer). The samples were transferred to the top chamber of a 30,000 MWCO cut-off filtration unit (Millipore, part MRCF030) and spun in a microcentrifuge at 14,000 r.c.f. for 10 min. An additional 200 μl of UA buffer was added, and the filter unit was spun at 14,000 r.c.f. for 15–20 min. The cysteine residues were alkylated using 100 μl of 50 mM iodoacetamide (Pierce, A39271) in UA buffer. Iodoacetamide in UA buffer was added to the top chamber of the filtration unit.

The samples were lyophilized at 550 r.p.m. for 30 min in the dark at room temperature using a thermomixer (Eppendorf). The filter was spun at 14,000 r.c.f. for 15 min, and the flow through was discarded. Unreacted iodoacetamide was washed through the filter with two sequential additions of 200 μl of 100 mM Tris-HCl buffer, pH 8.5 containing 5 M urea and 0.2% DCA, and the samples were centrifuged at 14,000 r.c.f. for 15–20 min after each buffer addition. The flow through was discarded after each buffer exchange centrifugation cycle. The urea buffer was exchanged with digestion buffer (DB; 50 mM ammonium bicarbonate buffer, pH 8, containing 0.2% DCA). Two sequential additions of DB (200 μl) with centrifugation after each addition to the top chamber was performed. The top filter units were transferred to a new collection tube, and 100 μl DB containing 1 μAU of LysC (Wako Chemicals, A39271) was added to the top chamber of a 30,000 MWCO cut-off filtration unit. The samples were gyrated at 550 r.p.m. for 30 min in the dark at room temperature using a thermomixer (Eppendorf). The filter was spun at 14,000 r.c.f. for 15 min, and the flow through was discarded. Unreacted iodoacetamide was washed through the filter with two sequential additions of 200 μl of 100 mM Tris-HCl buffer, pH 8.5 containing 5 M urea and 0.2% DCA, and the samples were centrifuged at 14,000 r.c.f. for 15–20 min after each buffer addition. The flow through was discarded after each buffer exchange centrifugation cycle. The urea buffer was exchanged with digestion buffer (DB; 50 mM ammonium bicarbonate buffer, pH 8, containing 0.2% DCA). Two sequential additions of DB (200 μl) with centrifugation after each addition to the top chamber was performed. The top filter units were transferred to a new collection tube, and 100 μl DB containing 1 μAU of LysC (Wako Chemicals, A39271) was added to the top chamber of a 30,000 MWCO cut-off filtration unit.
A pressure sensor was inserted into the cisterna magna. A baseline measure was done in saline solution. The capillary was left in place in the cisterna magna for 3 min. Intracranial pressure was recorded using FISO Evolution software (v.2.2.0.0). Respiratory rates were also measured using this method (breaths per min).

Western blots of isolated brain blood vessels

Brains were homogenized in a 2 ml tissue grinder in 1.5 ml of microvesSEL isolation buffer (MIB; 15 mM HEPES, 147 mM NaCl, 4 mM KCl, 3 mM CaCl2, and 12 mM MgCl2), containing a cocktail of protease inhibitors (complete, mini, EDTA-free protease inhibitor cocktail, Sigma-Aldrich).

Isolated vessels were lysed in RIPA buffer (Bioworld) containing protease inhibitors, and protein concentration was determined using a bicinchoninic acid assay kit (Pierce). Thirty micrograms of protein was separated by PAGE on 7.5% Mini-Protein TGX precast protein gels (Bio-Rad) and transferred onto nitrocellulose membranes. Ponceau S (Sigma-Aldrich) was used to confirm loading of equal amounts of protein and to monitor the transfer procedure. After blocking with blocking buffer (TBS 50 mM Tris, 150 mM NaCl, pH 7.6) containing 1% Tween-20 and 5% milk, the membranes were probed overnight (4°C) with a primary antibody (collagen IV antibody 134001, Bio-Rad, 1:500) diluted in blocking buffer. Membranes were rinsed in TBS containing 0.1% Tween-20 and incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (donkey anti-goat IgG (Abcam ab97120, 1:10,000) diluted in blocking buffer.

Behavioural tests

Behavioural tests were conducted at least 1 h after the dark cycle started. Behavioural tests were conducted in the following sequence: elevated plus maze → open-field test → three-chamber social-interaction test → forced-swim test (group 1) or cued-fear conditioning test (group 2). Each behavioural test was conducted with 50 dB white noise and at least 2 days apart to prevent stress. Experimental and social target mice were handled for 3 days before starting the first experiment. Before any behavioural tests, cages were located in a dark room with 50 dB white noise for 30 min.

Three-chamber social-interaction test. The size of the three-chambered apparatus was 40 × 20 × 26 cm (width, height, and depth, respectively), with a centre chamber that was 12 cm wide and side chambers that were 14 cm wide. Illuminance was kept at 50 lux. In the first session, the mouse could freely move around the entire three-chambered apparatus with two small containers in the left or right corner for 10 min (session 1). The mouse was then gently confined in the centre chamber while a new ‘object’ and a WT stranger mouse, ‘stranger 1’ (aged-matched C57BL/6J strain), was placed in one of the two plastic containers. The subject mouse was then allowed to freely explore all three chambers for 10 min (session 2). In the third session, the subject mouse was again gently guided to the centre chamber while the object was replaced with a WT ‘stranger 2’ mouse. The subject mouse again freely explored all three chambers for 10 min (session 3). Object and stranger exploration was defined by the nose of the subject mouse being oriented towards the target and coming within 2 cm of it as measured by EthoVision XT 15 (Noldus).
Open-field test. Mice were placed in an open-field box (35 × 35 × 35 cm) and recorded with a video camera for 60 min. The centre zone line was 9 cm apart from the edge. The testing room was illuminated at 0 lux. Mice movements were analysed using EthoVision XT 15 (Noldus).

Elevated plus maze. The elevated plus maze consisted of two open arms, two closed arms (for all arms, dimensions were 35 × 7 cm) and a centre zone, and was elevated to a height of 1 m above the floor. The illumination of the closed arm was 80 lux, whereas the open arm was 120 lux. Mice were placed in the centre zone and allowed to explore the space for 8 min. Data were analysed using EthoVision XT 15 (Noldus).

Forced-swim test. Mice were placed into a 4-litre beaker that was three-quarter filled with tap water and recorded with a video camera on the top and side for 5 min. Water temperatures were kept at 20–21 °C, and the room was at 120 lux. Behaviour was manually analysed.

Cued- fear conditioning test. On the first day, mice were placed in the fear conditioning chamber (Ugo Basile). Before starting the experiment, 70% ethanol was sprayed once in the chamber. After 2 min of acclimation in white noise and the experimental condition, a 1 kHz tone was applied for 20 s accompanied by 0.7 mA electric shock in the last 2 s. After 1 min of waiting time, tone-to-waiting was repeated three times in total. After repetition, mice were taken out of the conditioning chamber and returned to their home cage. On the second day (24 h after), the chamber was wiped with vanilla-flavoured oil, and the floor was exchanged with a grey-coloured plastic plate. Mice were placed in the chamber. After 2 min of acclimation to white noise, a 1 kHz tone was applied for 1 min, followed by 2 min of white noise. Mice were returned to the home cage. After 7 days of conditioning, the protocol for the second day was repeated. The data were analysed using activity parameters of EthoVision XT 15 (Noldus). Freezing time was measured during the waiting time (for the first day) and during the cue (for the second and eighth day).

Tissue collection and processing
Mice were anaesthetized with a KX cocktail, the head was shaved and the skin from the neck was removed using hooks to expose the dCLNs. Live imaging of the dCLNs was done using an intraperitoneal injection of euthasol (10% v/v in saline, 250 μl) and transcardially perfused with PBS containing 10 U ml−1 heparin. In some experiments, mice received an intravenous injection of lectin (30 μl; Dylight 649 labelled Lycopersicon Esculentum; Fisher Scientific, DL-1178) 5 min before perfusion. After removal of the skin, muscles and mandibles, the head was drop-fixed in 4% PFA for 24 h. Then, the skull caps (skull and attached dorsal dura mater) were detached and brains were kept in 4% PFA for an additional 24 h (48 h in total). When collected, the dCLNs were drop-fixed in 4% PFA for 12 h. After fixation, the tissues were cryoprotected with 30% sucrose solution and frozen in Tissue-Plus OCT compound (Thermo Fisher Scientific). Brains were sliced (100-μm-thick sections) with a cryostat and kept in 24-well plates filled with PBS at 4 °C. The dCLNs were sliced (30-μm-thick sections) and collected on gelatin-coated slides. In one experiment, whole brains were post-fixed with 4% PFA, then washed with PBS and were directly stained and imaged by stereomicroscopy.

Ex vivo stereomicroscopy imaging
Mice received a lethal intraperitoneal injection of euthasol (10% v/v in saline, 250 μl) and transcardially perfused with PBS containing 10 U ml−1 heparin. After removal of the skin, muscles and mandibles, the head was drop-fixed in 4% PFA for 24 h. Then, the skull caps (skull and attached dorsal dura mater) were detached and brains were kept in 4% PFA for an additional 24 h (48 h in total). The whole brains were then placed on a Petri dish and imaged by stereomicroscopy. For OVA measurements, whole brains were imaged using the following parameters: CY5 channel: zoom = 0.78, exposure time = 2 s. Quantification of OVA coverage at the MCA level was done using the following parameters: CY5 channel: zoom = 5, exposure time = 500 ms. For bead or pHrodo experiments, brains were placed on the side, and quantifications were done using the following parameters: GFP or CY5 channels: zoom = 5, exposure time = 250 ms or 500 ms, respectively.

Light sheet microscopy
For whole brain imaging, the vDISCO method was used to clear the brain46. In brief, mice received an i.c.m. injection of OVA (5 μl). One hour later, mice received an intravenous injection of lectin (30 μl) and were perfused 5 min later. Whole brain was post-fixed in 4% PFA for 24 h, then permeabilized and cleared using vDISCO protocol with passive tissue immersion. Brains were immersed in ethyl cinnamate (Sigma-Aldrich, W243019) and placed in chambered coverglass (Thermo Fisher, 153560) for light sheet imaging (LaVision BioTec).

IHC, imaging and quantifications
Tissues were rinsed in PBS and incubated in PBS 0.25% Triton X-100 with the following appropriate primary antibodies: rat anti-mouse CD206 (Bio-Rad MCA2235; 1:500); goat anti-mouse IBA1 (Abcam, ab5076; 1:500); Armenian hamster anti-mouse CD31 (Millipore Sigma, MAB1398Z; clone 2H8, 1:200); rabbit anti-mouse AQP4 (Millipore Sigma, A5971; 1:500); eFluor 660 conjugated anti-mouse LVYE1 (eBioScience, clone ALY7; 1:200); Alexa Fluor 347 conjugated anti-mouse I-A/I-E (MHCI; BioLegend, 107650; 1:200); rabbit anti-mouse laminin (Abcam, ab7463; 1:500); rabbit anti-mouse collagen IV (EMD Millipore, AB756P; 1:500); FITC-conjugated anti-mouse αSMA (Sigma Aldrich, F3777; 1:200); goat anti-mouse CD13 (R&D Systems, AF2335; 1:200); and rabbit anti-mouse Aβ (Cell Signaling Technology, 8243S, D54D2; 1:400). Sections were washed with a PBS solution containing 1:40,000 4’,6-diamidino-2-phenylindole (DAPI) and mounted on Superfrost Plus slides (Fisher Scientific) and coverslipped with Aqua-Mount (Lerner) or ProLong Gold (Thermo Fisher). Slices were acquired using wide-field (Olympus, VS200-S6) or confocal (Leica, DM-8 Stella-ris) microscopes. Quantitative analyses of imaging measurements were performed using the Fiji package for ImageJ software. For brain tracer coverage measurements, six sections per mouse were used and manually thresholded to match observed signals. All six images were quantified as area of signal/total area (DAPI coverage) and averaged to obtain one value per mouse. For ECM coverage, whole brain sections, cortical regions or lectin αSMA larger vessels (two whole sections per mouse) were manually thresholded and quantified as area of signal/total area.

In vivo fluorescent tracer dynamics evaluation
Mice were anaesthetized with a KX cocktail, the head was shaved and mice were placed on a stereotaxic frame. A lateral incision was made between the right eye and the right ear. The temporalis muscle was gently separated from the temporal bone. The surface of the skull was cleansed with a cotton bud. After i.c.m. injection of the fluorescent tracer, mice were positioned on a heating pad on their side to expose the right temporal bone under a stereomicroscope (Leica, M205 FA). The average time between the i.c.m. injection and the first image was 2 min. Mice were imaged over 1 h (240 frames in total; 4 frames per min). At the end of the imaging session, mice were euthanized using euthasol and tissues were collected for further analyses. For one experiment, mice were anaesthetized with KX or isoflurane (induction at 4.5% and continuously exposed at 0.75–2%). The fold increase was measured by calculating the ratio between the value divided by the minimal value.

For in vivo dCLN imaging, mice were anaesthetized with a KX cocktail, and the fur of the neck was shaved. After receiving an i.c.m. injection of OVA (5 μl), mice were placed on supine position, the skin from the neck was incised and retracted using hooks to expose the dCLNs. Live imaging was done under the stereomicroscope, as previously described (average of 10 min between the i.c.m. injection and the beginning of the imaging).
Mice were anaesthetized by a bolus of 4% isoflurane and medical air and maintained in 1–2% isoflurane during the surgical procedure. After fixing the head in a stereotaxic frame, a longitudinal incision was exposed to the skull. A small cranial window was made to be able to image brain blood vessels. Mice were sutured and allowed to recover after surgery in a clean cage. On the same day, mice were anaesthetized with 4% isoflurane and were transferred to the photoacoustic microscope, restrained using a nut and medical air was used for inhalation. The imaging session started 20 min after the mouse woke up. Thirty seconds after the acquisition started (average of 5,000 frames per mouse, frequency = 12 Hz), mice received a mixture of 10% CO₂ in medical air for 5 min. The vessel diameter fold-increase was measured by calculating the ratio between the measured values and the average of the first thousand values (before CO₂ challenge).

In vivo photoacoustic microscopy

Mice were anaesthetized in a prone position on the MRI device (7 Tesla MRI, Bruker Biospin). T2-weighted sequences were taken through the mouse head using the following parameters: repetition time = 3,000 ms, time to echo = 139 ms, field of view = 26 × 20.5 mm, slice thickness = 0.13 mm, number of slices = 160 and number of excitations = 3 (total acquisition = 16 min per mouse).

In vivo whisker stimulation

Mice were anaesthetized using a cocktail and placed in a stereotaxic frame. The body temperature was constantly monitored and adjusted using a heating pad. Whiskers on the right side of the mice were cut to about 0.5 cm. A longitudinal incision was made to expose the skull, and a thinned-skull window was made at the left barrel cortex to enable imaging of the distal part of the MCA. Mice were then placed under a stereomicroscope for imaging. Mechanical whisker stimulation was performed for 10, 5, or 10 s after the beginning of the imaging session. An average of ten videos were made per mouse (five with stimulations and five without stimulation), using the following parameters: bright-field channel; ×16 zoom; exposure time: 200 ms (5 frames per s). Heart rates (beats per min) and basal arterial pulsations (pulsatile amplitude per vessel diameter) were measured using the videos without whisker stimulations.

Flow cytometry

Mice received a lethal intraperitoneal injection of euthasol (10% v/v in saline, 250 μl) and were transcardially perfused with PBS containing 10 U/ml heparin. Lateral choroid plexuses were removed from half or whole brains. Brains were then digested for 40 min at 37 °C with 1.4 U/ml of collagenase VIII and 35 U/ml of DNase I in DMEM. The cell pellets were washed and resuspended in FACS buffer and stained with the following antibodies (1:200 dilution; eBioscience): rat anti-mouse Ly6C (BioLegend, 128033, BV510); rat anti-mouse XCR1 (BioLegend, 147100, AF488); rat anti-mouse CD11b (BioLegend, 741242, BV661); rat anti-mouse MHCII (BioLegend, 107641, BV650). Cell viability was determined using DAPI staining.

Magnetic resonance imaging (MRI)

Immediately after i.c.m. injection of Dotarem (a gadolinium-based MRI contrast agent; 0.754 kDa; 5 μl) under KX anaesthesia (average time between i.c.m. injection and start of the MRI acquisitions: 2 min), mice were placed in a prone position on the MRI device (9.4 Tesla MRI, Bruker Biospin). During the imaging session, 0.5–0.8% isoflurane was provided through a nose mask to prevent mice from waking up. Body temperature and respiratory rates were monitored during imaging. Isoflurane levels were adjusted with respiratory rates, and body temperature was controlled using a heating pad. A series of post-contrast T1 Fast Low Angle Shot (FLASH) 3D weighted images were taken through the head with the following parameters: repetition time = 30 ms; echo time = 8 ms; number of echo images = 1; number of averages = 1; number of repetitions = 12; scan time = 272,640 ms per sequence (4 m 54 s); flip angle = 20, field of view = 160 × 160 × 80 μm with a 128 × 128 × 64 matrix; spatial resolution = 125 × 125 × 125 μm (8 pixels per mm; voxel size = 0.125 mm³), number of slices = 64; receiving coil 4 elements RF ARR 400 1H M. The total acquisition time was about 1 h per mouse (4 m 54 s ×12 sequences per mouse). To calculate the volume of ventricles, mice were placed on supine position on the MRI device (7 Tesla MRI, Bruker Biospin). T2-weighted sequences were taken through the mouse head using the following parameters: repetition time = 3,000 ms, time to echo = 139 ms, field of view = 26 × 20.5 mm, slice thickness = 0.13 mm, number of slices = 160 and number of excitations = 3 (total acquisition = 16 min per mouse).

Electron microscopy

Mice received a lethal dose of euthasol by intraperitoneal injection and were transcardially perfused with warm Ringer’s solution followed by perfusion with warm fixative containing 2.5% glutaraldehyde and 2% paraformaldehyde in 0.15 M cacodylate buffer (2 mM CaCl₂, at pH 7.4). Brains were transferred to a fixative solution and allowed to fix overnight at 4 °C. Brains were then rinsed in cacodylate buffer 3 times for 10 min and fixed in 1% osmium tetroxide and 1.5% potassium ferrocyanide in cacodylate buffer for 1 h. Brains were then washed in ultrapure water 3 times for 10 min and stained in 1% thionycarbohydrazide solution for 1 h, followed by 1 h staining in aqueous 2% osmium tetroxide. Brains were then rinsed in ultrapure water 3 times for 10 min and stained overnight in 1% uranyl acetate at 4 °C. The brains were then washed in ultrapure water 3 times for 10 min and stained with 20 mM lead aspartate at 60 °C for 30 min. After staining was complete, samples were washed in ultrapure water, dehydrated in a graded acetone series (50%, 70%, 90%, 100% three times) for 10 min in each step, and infiltrated with low viscosity resin (resin for electron microscopy, negative stain). Samples were flat embedded and cured in an oven at 60 °C for 48 h. After resin curing, 70-nm-thick sections from brain cortex were prepared on copper grids, post-stained with uranyl acetate and Reynold’s lead and imaged on a scanning electron microscope (Zeiss Merlin FE-XEM) using the following parameters: voltage = 5.00 kV; probe current = 3.0 nA; and WD = 6.9 mm.

MMP activity assay

The left hemisphere of the brain was homogenized in 6 volumes of PBS using a mini bead beater (Sigma Aldrich) and 2.3 mm diameter zirconia/silica beads (Biospec). The homogenate was then centrifuged in a microcentrifuge (Eppendorf) at 14,000 r. c. f. for 20 min at 4 °C. Protein (100 μg) from the supernatant (representing the soluble fraction of the brain) was incubated with 25 μm of the quenched fluorescent MMP substrate BML-P128-0001 (Enzo Life Sciences) in PBS and incubated at 37 °C for 15 min. Afterwards, the fluorescence of the cleaved fluorescent product was measured using a BioTek Synergy H1 plate reader (excitation/emission = 340 nm/440 nm).

RNA-seq

Mus musculus Dil-liposome leptomeningeal scRNA-seq. Sample preparation. Fifteen mice (3-month-old males; C57BL6/J) received an i.c.m. injection of 5 μl (23 mg ml⁻¹) of Dil-liposomes (Liposoma, I-005). Mice were perfused with heparinized PBS and tissues were collected the next day. Subdural meninges were gently collected from the brains and were disected in FACS buffer and digested in a digestion solution containing 1:50 collagenase VIII, 1:50 DNase, 1:50 FBS in FACS buffer for 15 min. Tissues were then washed through 70-μm strainers in 50-ml
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tubes containing FACS buffer and 10% FBS to stop enzymatic digestion. After centrifugation, the supernatant was removed and cells were resuspended in FACS buffer and transferred to a V-bottom plate. Cells were sorted, and only live Dill cells were used for sequencing.

**Single-cell data pre-processing.** Reads were aligned to the mm10 genome using the Cellranger software pipeline (v.3.0.2) provided by 10x genomics. The resulting filtered gene-by-cell matrix of unique molecular identifier (UMI) counts was read in R using the read10xCounts function from the DropetUtils package. Filtering was applied to remove low-quality cells by excluding cells expressing fewer than 200 or greater than 5,000 unique genes, having fewer than 1,000 or greater than 30,000 UMI counts, as well as cells with greater than 20% mitochondrial gene expression. Expression values for the remaining cells were then normalized using the scan and scatter packages. The resulting log values were transformed to the natural log scale for compatibility with the Seurat (v.3) pipeline.54–47

**Dimensionality reduction and clustering.** The filtered and normalized matrix was used as input to the Seurat pipeline, and cells were scaled across each gene before the selection of the top 2,000 most highly variable genes using variance stabilizing transformation. Principal components analysis was conducted, and an elbow plot was used to select the first ten principal components (PCs) for t-distributed stochastic neighbour embedding (tSNE) analysis and clustering. Shared nearest neighbour (SNN) clustering optimized with the Louvain algorithm, as implemented by the Seurat FindClusters function, was performed before manual annotation of clusters based on the expression of canonical gene markers. Macrophages were then subset out, rescaled and clustered as above with the first 21 PCs and a resolution of 0.3.

**M. musculus CLO depletion scRNA-seq. Sample preparation.** To deplete PBMs, mice received an i.c.m. injection of CLO-loaded liposomes (5 μl; 5 mg ml⁻¹; Fisher Scientific; CLD-8901). The control group consisted of mice that received an i.c.m. injection of PBS-loaded liposomes. One week later, mice received a lethal intraperitoneal injection of euthasol (10% v/v in saline, 250 ml) and transcardially perfused with PBS containing 10 U ml⁻¹ heparin. Lateral choroid plexuses were gently removed, then whole brains were digested, myelin was removed and brain was stained with CD13 (to stain for mural cells), CD31 (endothelial cells) and CD45 (immune cells). Stromal cells (that is, endothelial plus mural cells) were then sorted and prepared for single-cell sequencing.

**Single-cell data pre-processing.** Reads were aligned to the mm10 genome using the Cellranger software pipeline (v.6.0.0) provided by 10x genomics. The resulting filtered gene-by-cell matrix of UMI counts was read in R using the read10xCounts function from the DropetUtils package. Filtering was applied to remove low-quality cells by excluding cells expressing fewer than 200 or greater than 7,000 unique genes, having fewer than 1,000 or greater than 50,000 UMI counts, as well as cells with greater than 15% mitochondrial gene expression. Expression values for the remaining cells were then normalized using the scan and scatter packages. The resulting log values were transformed to the natural log scale for compatibility with the Seurat (v.3) pipeline.45–47

**Dimensionality reduction and clustering.** The filtered and normalized matrix was used as input to the Seurat pipeline, and cells were scaled across each gene before the selection of the top 2,000 most highly variable genes using variance stabilizing transformation. Principal components analysis was conducted, and an elbow plot was used to select the first 10 PCs for tSNE analysis and clustering, SNN clustering optimized with the Louvain algorithm, as implemented by the Seurat FindClusters function, was performed before manual annotation of clusters based on the expression of canonical gene markers.

**Cell–cell interaction analysis.** To evaluate potential cell–cell or ligand–receptor interactions in an unbiased way, the RNA Magnet package was utilized with membrane, ECM, as well as both, ligand–receptor pairs queried and all vascular network cell types (arterial, capillary and venous endothelial cells, pericytes and VSMCs) included as anchors for RNAMagnetAnchors. Signalling interactions were also investigated with RNAMagnetSignaling, and the top signalling pair molecules were examined for both endothelial cells and mural cells with each other cell type present in the dataset. Macrophages were then subset out, rescaled and clustered as above with the first seven PCs and a resolution of 0.3.

**M. musculus 5xFAD scRNA-seq. Sample preparation.** Mice received a lethal intraperitoneal injection of euthasol (10% v/v in saline, 250 ml) and transcardially perfused with PBS containing 10 U ml⁻¹ heparin. Cortices were gently dissected in PBS, digested and stained after myelin removal with CD13 (to stain for mural cells), CD31 (endothelial cells) and CD45 (immune cells). Stromal cells (that is, endothelial and mural) and immune cells were then sorted and prepared for single-cell sequencing.
Single-cell data pre-processing. Reads were aligned to the mm10 genome using the Cellranger software pipeline (v.6.0.0) provided by 10x genomics. The resulting filtered gene-by-cell matrix of UMI counts was read in R using the read10xCounts function from the DropletUtils package. Filtering was applied to remove low-quality cells by excluding cells expressing fewer than 200 or greater than 7,500 unique genes, having fewer than 1,000 or greater than 40,000 UMI counts, as well as cells with greater than 25% mitochondrial gene expression. Expression values for the remaining cells were then normalized using the scan and scater packages. The resulting log values were transformed to the natural log scale for compatibility with the Seurat (v.3) pipeline. Dimensionality reduction and clustering. The filtered and normalized matrix was used as input to the Seurat pipeline, and cells were scaled across each gene before the selection of the top 2,000 most highly variable genes using variance stabilizing transformation. Principal components analysis was conducted, and an elbow plot was used to select the first 22 PCs for tSNE analysis and clustering. SNN clustering optimized with the Louvain algorithm, as implemented by the Seurat FindClusters function, was performed before manual annotation of clusters based on the expression of canonical gene markers.

Cell-cell interaction analysis. To evaluate potential cell–cell or ligand–receptor interactions in an unbiased way, the RNA Magnet package was utilized with membrane, ECM, as well as both, ligand–receptor pairs queried and all vascular, stromal and mural cell types (arterial, capillary and venous endothelial cells, ependymal cells, fibroblasts, pericytes and VSMCs) included as anchors for RNA Magnet Anchors. Macrophages were then subset out, rescaled and clustered as above with the first 14 PCs and a resolution of 0.6.

Homo sapiens single-nucleus RNA-seq. Sample preparation. The Neuropathology Core of the Knight Alzheimer’s Disease Research Center and the Dominantly Inherited Alzheimer Network (DIAN) provided the parietal lobe tissue of postmortem brains for each sample. These samples were obtained with informed consent for research use and were approved by the review board of Washington University in St Louis. AD neuropathological changes were assessed according to the criteria of the National Institute on Aging–Alzheimer’s Association (NIA–AA). From the 60 frozen human parietal lobes, approximately 500 mg of tissue was cut and weighed on dry ice using sterile disposable scalps. The parietal tissue was homogenized in ice-cold homogenization buffer (0.25 M sucrose, 150 mM KCl, 5 mM MgCl₂, 20 mM tricine-KOH pH 7.8, 0.15 mM spermine, 0.5 mM spermidine, EDTA-free protease inhibitor and recombinant RNase inhibitors) with a dounce homogenizer. Homogenates were centrifuged for 5 min at 500g, at 4 °C, to pellet the nuclear fraction. The nuclear fraction was mixed with an equal volume of 50% iodixanol and added on top of a 35% iodixanol solution for 30 min at 10,000g, at 4 °C. After myelin removal, the nuclei were collected at the 30–35% iodixanol interface. Nuclei were resuspended in nuclei wash and resuspension buffer (% BSA and recombinant RNase inhibitors in PBS) and pelleted for 5 min at 500g and 4 °C. Nuclei were passed through a 40-μm cell strainer to remove cell debris and large clumps. Nuclei concentration was manually determined using DAPI counterstaining and a haemocytometer. Nuclei concentration was adjusted to 1,200 nuclei per μl and processed immediately following the 10x Genomics Single Cell Protocol instructions. We generated single-nucleus RNA-seq libraries using a 10x Chromium single cell reagent Kit v3 for 10,000 cells per sample and sequenced 50,000 reads per cell from 31 frozen human parietal lobes.

Single-cell data pre-processing. We prepared a pre-mRNA reference according to the steps detailed by 10x Genomics based on the GRC38 (3.0.0) reference, and reads were aligned to the using the Cellranger software pipeline (v.3.0.2). The resulting filtered gene-by-cell matrices of UMI counts for each sample were read in R using the read10xCounts function from the DropletUtils package. Filtering was applied to remove low-quality cells by excluding cells expressing fewer than 500 or greater than 10,000 unique genes, having fewer than 2,000 or greater than 100,000 UMI counts, as well as cells with greater than 25% mitochondrial gene expression. Samples were then randomly assigned to one of five cohorts and individually processed to screen for the possible presence of perivascular macrophages. Expression values for the remaining cells in each cohort were merged by gene symbol into one dataframe and normalized using the scan and scater packages. The resulting log values were transformed to the natural log scale for compatibility with the Seurat (v.3) pipeline. Each was then scaled across each gene before the selection of the top 2,000 most highly variable genes using variance stabilizing transformation. Principal components analysis was conducted, and an elbow plot was used to select PCs for tSNE analysis and clustering. SNN clustering optimized with the Louvain algorithm, as implemented by the Seurat FindClusters function, was performed before manual annotation of clusters based on the expression of canonical gene markers. After removal of cells identified as neurons, oligodendrocytes, oligodendrocyte precursor cells and astrocytes, the remaining cells were split by original sample identity.

Integration, dimensionality reduction and clustering. Reference samples were chosen as those with more than 500 cells per sample and were prepped for integration utilizing the SCT normalization provided by Seurat with functions SelectIntegrationFeatures and PrepSCTIntegration. Sample integration was then performed with FindIntegrationAnchors specifying a k.filter = 100 and the reference samples determined above, and IntegrateData. Principal components analysis was conducted, and an elbow plot was used to select the first 30 PCs for tSNE analysis and clustering. SNN clustering optimized with the Louvain algorithm, as implemented by the Seurat FindClusters function, was performed before manual annotation of clusters based on the expression of canonical gene markers.

Differential expression. For analysis of differentially expressed genes between conditions, each cluster was filtered to include genes that had at least 5 transcripts in at least 5 cells, then the top 2,000 highly variable genes were determined and included for further analysis using the SingleCellExperiment modelGeneVar and getTopHVGs functions. After filtering, observational weights for each gene were calculated using the ZINB-WaVE zinbFit and zinbwave functions. These were then included in the edgeR model, which was created with the glmfit function, by using the glmWeighteddf function. Results were then filtered using a Benjamini–Hochberg-adjusted P value threshold of less than 0.05 as statistically significant.

Pathway enrichment. Over-representation enrichment analysis with Fisher’s exact test was used to determine significantly enriched Gene ontology terms (adjusted P < 0.05) for the sets of significantly differentially expressed genes. For each gene set, genes were separated into upregulated and downregulated, and separately, the enrichGO function from the clusterProfiler package was used with a gene set size set between 10 and 500 genes and P values adjusted using Benjamini–Hochberg correction.

Statistical analyses and reproducibility. All data are presented as the mean ± s.e.m. All the experiments (except single-cell and single-nuclei RNA-seq data) were repeated independently at least two times (biological replicates). Statistical significance was determined using two-tailed paired Welch’s t-test (nonparametric) when comparing two independent groups or by paired t-test when comparing values from the same group. For comparisons of multiple factors, one-way or two-way analysis of variance (ANOVA) with appropriate multiple-comparisons tests were used. Statistical analyses were performed using Prism 9.0 (GraphPad software). Exact P values are all provided in figures. We provide all raw data and the statistical analyses as a PRISM file in Supplementary Data 1. All experiments were done blinded, and groups were revealed only after all the analyses were performed.
Acknowledgements We thank C. Smith for editing the manuscript; S. Blackburn, N. Al Hamadani, K. Wang and E. Griffin for animal care; S. Brophy for laboratory management; all the members of the Kipnis Laboratory for their valuable comments during numerous discussions of this work; all the members of the Washington University Center for Cellular Imaging core (WUCCI) for their valuable contribution of electron microscopy imaging; staff at the University of Virginia Flow Cytometry Core and from the Sequencing Core for their help with cell sorting and sequencing; all the members of the Washington University Small Animal MR Imaging Facility and the University of Virginia Molecular Imaging Core Facility for their help in MRI. We acknowledge the expert technical assistance of Y. Mi, P. Edmann-Gilmore, A. Davis and R. Connors for the CSF proteomics experiment performed at the Washington University Proteomics Shared Resource (WU-PSR), and R. Reid Townsend (Director) and R. Sprung and T. Zhang (Co-directors); the staff of the Neuropathology Core and other personnel of the Charles F. and Joanne Knight Alzheimer’s Disease Research Center (ADRC), and the altruism of the participants and their families and contributions of the Knight ADRC and DIAN research support staff at each of the participating sites for their contributions to this study. This work was supported by grants from the National Institutes of Health/National Institute on Aging (AG034113, AG037486, AG078106), the Cure Alzheimer’s Fund and the Ludwig Foundation to J.K.; AG057777 and AG067764 to O.H.; and AG062734 to C.M.K. O.H. is an Archer Foundation Research Scientist. The WU-PSR is supported in part by the WU Institute of Clinical and Translational Sciences (NCATS UL1 TR000448), the Mass Spectrometry Research Resource (NGMS P41 GM103422; P24GM136766) and a Sitemap Comprehensive Cancer Center Support grant (NCI P30 CA019842). The Neuropathology Core and the Charles F. and Joanne Knight ADRC are supported by P30 AG066444, P01AG026276 and P01AG053991. Data collection and sharing for this project was supported by the DIAN (UF140032438) funded by the National Institute on Aging (NIA), the German Center for Neurodegenerative Diseases (DZNE), Raul Carrea Institute for Neurological Research (FLENI), partial support by the Research and Development Grants for Dementia from Japan Agency for Medical Research and Development, AMED, and the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI). This manuscript has been reviewed by DIAN Study investigators for scientific content and consistency of data interpretation with previous DIAN Study publications. The illustrations of the mice and MRI are freely available from Server Medical Art (https://smart.server.com). The brain cartoon and the summary illustration were created by the authors.

Author contributions A.D. designed and performed experiments, analysed and interpreted data, created the figures and wrote the manuscript. S.D. and S.E.S. designed and performed experiments, analysed and interpreted data. F.Z. and S.H. performed the photoacoustic imaging experiment. Z.P. performed the live imaging on dCLNs. S.B. assisted in experiments and data analyses. J.R. and T.M. provided intellectual contribution and assisted in experiments. T.D. designed and performed experiments, analysed and interpreted data. F.Z. and S.H. performed the photoacoustic imaging experiment. Z.P. performed the live imaging on dCLNs. S.B. assisted in experiments and data analyses. J.R. and T.M. provided intellectual contribution and assisted in experiments. T.D. performed the mouse scRNA-seq data analyses, analysis of human single-nucleus RNA-seq data and participated in methods writing. K.K. performed the behavioural experiments and analyses. O.H. supervised and interpreted the scRNA-seq data analysis. C.M.K., R.J.B., R.P., M.F., L.F. and O.H. provided intellectual contribution and assisted in experiments. J.R. and T.M. provided intellectual contribution and assisted in experiments. Z.P. performed the live imaging on dCLNs. S.B. assisted in experiments and data analyses. J.R. and T.M. provided intellectual contribution and assisted in experiments. T.D. performed the mouse scRNA-seq data analyses, analysis of human single-nucleus RNA-seq data and participated in methods writing. K.K. performed the behavioural experiments and analyses. O.H. supervised and interpreted the scRNA-seq data analysis. C.M.K., R.J.B., R.P., M.F., J.C. and P.S. generated the datasets for the DIAN network. G.J.R. provided LyveFcre mice and intellectual contribution. I.S. assisted with animal surgeries and blinded data analyses and quantifications. J.K. designed the experiments, provided resources and intellectual contribution, oversaw data analysis and interpretation, and wrote the manuscript.

Competing interests J.K. is a scientific advisor and collaborator with PureTech. J.K. and A.D. are holding provisional patent applications related to the findings described herein. R.J.B co-founded and is on the scientific advisory board of C2N Diagnostics. C2N Diagnostics has licensed certain anti-tau antibodies to AbbVie for therapeutic development. He receives research support from Biogen, Eisai and the DIAN-TU Pharma Consortium. He is also an advisor to Amgen and Hoffman La-Roche.

Additional information The online version contains supplementary material available at https://doi.org/10.1038/s41586-022-05397-3.

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Peer review information Nature thanks Kayash Movahed and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

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Code availability Custom code used to analyse the mRNA sequencing data is freely available at https://doi.org/10.5281/zenodo.7047054.
Extended Data Fig. 1 | PBMs are distinct from microglia and sample CSF and ISF. **a**, CD206+ PBMs (cyan) are easily distinguishable from IBA1+ microglia (yellow) and are located at the vicinity of i.v. lectin+ large blood vessels (red). Scale bar, 100 μm. **b**, PBMs are located outside of the brain vasculature, in the perivascular space. **c**, Quantification of whole brain sections showing spatial distribution of PBMs through both perivascular space (PVS) and leptomeninges (LM). Scale bar, 20 μm. n = 5 mice. **d**, Gating strategy for PBM detection. PBMs were defined as DAPI−CD45−TCRβ−CD19−CD11b+CD64hiF4/80hiCD206+ cells. PBMs can be divided in subtypes using MHCII and CD38. **e**, WT mice received an i.c.m. injection of Alexa-647 conjugated ovalbumin (OVA; 45 kDa; 1 mg/ml; 5 μl). One hour after OVA injection, mice received an i.v. injection of Alexa-594 conjugated lectin (30 μl) and were perfused five minutes later. Maximum projection image obtained by light sheet microscopy from a cleared mouse brain showing brain OVA (magenta) distribution at the vicinity of i.v.-injected lectin+ blood vessels (cyan). Scale bar, 1mm. **f**, WT mice received an i.c.m. injection of Alexa-647 conjugated ovalbumin (OVA; 45 kDa; 1 mg/ml; 5 μl). Mice were perfused one hour after OVA injection. Representative stereomicroscopy images showing whole brain OVA distribution from the distal part of the middle cerebral artery (MCA), and quantification of both perivascular and cellular OVA distribution. Scale bars, 1mm and 200 μm (inset). n = 6 mice. **g**, Experimental schematic: WT mice received an i.c.m. injection of FITC Dextran (FITC-Dex; 4 kDa; 10 mg/ml; 5 μl) and brain were harvested one hour later. Brain coronal sections were stained for anti-CD206 (cyan) and DAPI. Scale bars, 2 mm and 50 μm (insets). **h**, Experimental schematic: WT mice received an intrastriatal (i.s.) injection of a cocktail containing 0.5 μl of FITC-Dex (10mg/ml; green) and 0.5 μl of OVA (1 mg/ml; magenta) and brains were harvested one hour later. Brains were then stained for anti-CD206 (cyan). Scale bars, 2 mm and 50 μm (insets). **i**, Mice received an i.s. injection of A488-OVA (green) and an i.c.m. injection of A647-OVA (magenta) one hour later. Mice were perfused one hour later (two hours after the i.s. injection). Some cells sampled both i.s. and i.c.m. OVAs. Scale bars, 2 mm and 100 μm (inset). All data are presented as mean values +/- SEM. The illustration of mice in **g** and **h** are from Servier Medical Art, CC BY 3.0.
Extended Data Fig. 2 | See next page for caption.
Extended Data Fig. 2 | Effect of PBM depletion. **a**, WT mice received an i.c.m. injection of clodronate-loaded liposomes (CLO) or PBS-loaded liposomes (PBS). Microglial cells were identified using anti-IBA1 staining (yellow). Cells that were not in the leptomeninges and CD206- were used for quantifications of cell numbers and Sholl analysis. Scale bars, 100 μm and 20 μm. n = 5 mice/group; two-tailed unpaired Welch’s t-test; repeated measures 2-way ANOVA with Geisser-Greenhouse correction. **b**, WT mice received an i.c.m. injection of Dil-liposomes (5 mg/ml; 5 μl) and mice were perfused 24 h later. Representative images showing Dil-liposome coverage in whole brains. Scale bar: 2mm. **c**, Representative images showing Dil-liposome (cyan) uptake by CD206+ PBMs (magenta) in leptomeninges (LM) co-stained for DAPI. Scale bars: 50 μm and 10 μm (inset). **d**, Experimental schematic: Twenty-four hours after i.c.m. injection of Dil-liposomes, leptomeninges were harvested, Dil-positive cells were sorted and single-cell RNA sequencing was performed. **e**, tSNE plot showing Dil-positive cells: monocytes, PBMs, granulocytes, migratory dendritic cells (migDCs), fibroblasts and NK/T cells. **f**, Single-cellRNA sequencing demonstrating 4 PBM clusters. **g**, Dot plots showing Mrc1, H2-Ab1, Cd74, Cd163 and Lyve1 gene expression in the 4 PBM subtypes. **h**, Volcano plot corresponding to down- and up-regulated genes comparing MHCIIhi vs. Lyve1hi PBMs. F-test with adjusted degrees of freedom based on weights calculated per gene with a zero-inflation model and Benjamini-Hochberg adjusted P values. **i**, GO Pathway analysis showing up- and down-regulated pathways comparing MHCIIhi vs. Lyve1hi PBMs. Over-representation test. **j**, Experimental schematic: WT mice received an i.c.m. injection of CLO or PBS liposomes. OVA was injected i.c.m. three days later and mice were perfused one hour later. **k**, Representative images showing CD206+ PBMs (cyan) on brain coronal section co-stained for DAPI, and corresponding quantification. Scale bar, 200 μm. n = 5 mice/group; two-tailed unpaired Welch’s t-test. **l**, Representative images and quantification of OVA coverage in whole brains one hour after OVA injection. Scale bar, 5 mm. **m**, Representative images and quantification of OVA coverage in brain coronal sections. Scale bar, 2 mm. For l and m: n = 5 mice/group; two-tailed unpaired Welch’s t-test. **n**, Representative images from non-superior sagittal sinus region of the dura mater three days after CLO treatment co-stained for anti-CD206 (cyan) and anti-CD31 (yellow) and corresponding quantifications. **o**, Representative images from the superior sagittal sinus region from the dura mater three days after CLO treatment co-stained for anti-CD206 (cyan) and anti-CD31 (yellow), and corresponding quantifications. For n and o: Scale bars, 200 μm; n = 5 mice/group; two-tailed unpaired Welch’s t-test. **p–u**, WT mice received an i.c.m. injection of CLO or PBS liposomes. Tissues were harvested one week later. **p**, Representative images of total Lyve1 (magenta) coverage in the dura mater co-stained for DAPI and corresponding quantification. Scale bar, 3 mm. **q**, High magnification images showing LYVE1 staining at the transverse sinus level co-stained for DAPI and corresponding quantification. Scale bar, 200 μm. **r**, High magnification images showing LYVE1 staining at the superior sagittal sinus level co-stained for DAPI and corresponding quantification. Scale bar, 200 μm. **s**, High magnification images showing CD206 staining (cyan) co-stained for anti-CD31 (yellow) and corresponding quantification. Scale bar, 500 μm. For p–s: n = 5 mice/group; two-tailed unpaired Welch’s t-test. **t**, Representative images from lateral choroid plexuses whole mounts co-stained for anti-CD206 (cyan), anti-CD31 (red) and DAPI with corresponding high magnifications and quantification. Scale bars, 2 mm and 200 μm (insets). n = 5 mice treated with PBS, and 6 mice treated with CLO; two-tailed unpaired Welch’s t-test. **u**, Representative images from deep cervical lymph nodes co-stained for anti-IBA1 (cyan) and DAPI and corresponding quantification. Scale bar, 200 μm. n = 10 mice/group; two-tailed unpaired Welch’s t-test. All data are presented as mean values +/- SEM. The illustrations of mice in **d** and **j** are from Servier Medical Art, CC BY 3.0.
Extended Data Fig. 3 | See next page for caption.
Extended Data Fig. 3 | CSF flow after PBM depletion. a, One week after PBM depletion, mice received an i.c.m. injection of FITC-Dextran (FITCdex; 4 kDa; 5 μl), brains were harvested one hour later and FITCdex (green) coverage was measured on coronal sections co-stained for DAPI. Representative images and corresponding quantifications are shown. Scale bar, 2 mm. n = 5 mice/group; two-tailed unpaired Welch’s t test. b, One week after PBM depletion, mice received an i.c.m. injection of Texas Red (3 kDa; 5 μl), brains were harvested one hour later and Texas Red (red) coverage was measured on coronal sections co-stained for DAPI. Representative images and corresponding quantifications are shown. Scale bar, 2 mm. n = 4 mice treated with PBS, and 5 mice treated with CLO; two-tailed unpaired Welch’s t test. c, One week after PBM depletion, mice received an intrastriatal injection of OVA (45 kDa; 1 μl) and brains were harvested one hour later. Representative images and corresponding quantifications are shown. Scale bar, 2 mm. n = 4 mice treated with PBS, and 5 mice treated with CLO; two-tailed unpaired Welch’s t test. d, One week after PBM depletion, mice received an intrastriatal (i.s.) injection of FITC-Dextran (FITCdex; 4 kDa; 1 μl) and brains were harvested one hour later. Representative images and corresponding quantifications are shown. Scale bar, 2 mm. n = 4 mice/group; Two-tailed unpaired Welch’s t-test. e, One week after CLO or PBS liposome injection, mice were anesthetized, and a glass capillary was inserted i.c.m. to collect CSF for proteomic analyses. f, Volcano plot corresponding to down- and up-regulated proteins in CSF comparing PBM-depleted and control mice. F-test with adjusted degrees of freedom based on weights calculated per gene with a zero-inflation model and Benjamini-Hochberg adjusted P values. g, Corresponding GO Pathway analysis showing down- and up-regulated pathways in PBM-depleted and control mice. Over-representation test. h, Sunburst plot representing the location of the upregulated CSF-derived neuronal/synaptic-related proteins after PBM depletion. i–k, Quantification of relative spectral counts for i, Clusterin (CLU); j, Apolipoprotein E (APOE) and k, Amyloid Precursor Peptide (APP). For e–k: n = 4 mice treated with PBS, and 5 mice treated with CLO; two-tailed unpaired Welch’s t test. m, Representative T2 images showing lateral ventricles (in hypersignal) before and after PBM depletion. n, Quantification of ventricle volume in mm³. n = 5 mice/group; one-way ANOVA with Tukey multiple comparisons test. o, Intracranial pressure was measured one (7d) and three (21d) weeks after PBM depletion. n = 5 mice treated with PBS, 7 mice treated with CLO at 7d; 6 mice treated with PBS, and 7 mice treated with CLO at 21 d; two-way ANOVA with Sidak’s multiple comparisons test. p, Sagittal view of a T1-FLASH 3D image showing Dotarem (0.754 kDa; 5 μl) accumulation in different brain compartments, including the olfactory bulbs (OB), the lateral ventricles (Lat vtl) and the middle cerebral artery (MCA). Scale bar, 3 mm. q, Representative T1-FLASH 3D images showing Dotarem distribution at the MCA level over time. Scale bar, 3 mm. r, Quantification of Dotarem signal fold increase over an hour. n = 5 mice treated with PBS, and 7 mice treated with CLO; repeated measures 2-way ANOVA with Geisser-Greenhouse correction. s, One week after CLO or PBS liposome injection, mice received an i.c.m. injection of OVA, and deep cervical lymph nodes (dCLNs) were harvested one hour later. t, Representative images showing OVA coverage on dCLN sections. Scale bar, 200 μm. u, Quantification of dCLN area. v, Quantification of OVA coverage. For u and v: n = 10 mice/group; two-tailed unpaired Welch’s t-test. w, One week after CLO or PBS liposome injection, mice received an i.c.m. injection of OVA and then placed in supine position under the stereomicroscope for dynamic imaging of OVA diffusion in the exposed lymph nodes. x, Representative images showing OVA coverage in dCLNs over time. Scale bar, 500 μm. y, Quantifications of both OVA influx (left) and efflux (right) over time. n = 3 mice treated with PBS, and 7 mice treated with CLO; repeated measures 2-way ANOVA with Geisser-Greenhouse correction. z, Proportion of mice that showed OVA outflow from dCLNs. All data are presented as mean values ± SEM. The illustrations of mice in e, s and w are from Servier Medical Art, CC BY 3.0.
Extended Data Fig. 4 | See next page for caption.
Extended Data Fig. 4 | In vivo recording of tracer diffusion at the middle cerebral artery (MCA) level and evaluation of the perivascular space.

A, Experimental schematic: one week after CLO or PBS liposome injection, mice were placed in a stereotaxic frame, the top and the right side of the head were shaved, the skin was incised, and the right temporalis muscle was gently removed. B, After cleaning the area with a cotton bud, mice received an i.c.m. injection of OVA and were immediately placed on their side under the stereomicroscope. C, Example of OVA distribution at the middle cerebral artery level (MCA) before and 20 min after injection. Scale bar, 5 mm. D, Inset of C. Higher magnification image showing OVA distribution one hour after injection. The tracer is located around the vessel, at the perivascular level. Scale bar, 1 mm.

E, Representative images showing OVA distribution over time. Scale bar, 1 mm. F, Quantification of OVA coverage over time. n = 5 mice/group; two-way ANOVA mixed-effects analysis (30 last min only). G, Representative images showing OVA distribution over time. Scale bar, 1 mm. H, Quantification of OVA coverage on brain coronal sections. Scale bars, 2 mm (left panels) and 1 mm (right panels). I, Representative images showing OVA distribution and quantification in whole brains and a zoom on the middle cerebral artery. Scale bars, 2 mm (left panels) and 1 mm (right panels). J, Representative images showing OVA coverage on brain coronal sections. Scale bar, 2 mm. For I and J, n = 5 mice/group; two-tailed unpaired Welch’s t test. K, One week after PBM depletion and one hour after i.c.m. injection of OVA, mice were perfused, brains were extracted, and OVA distribution was analyzed on coronal sections stained for DAPI, and corresponding quantification of OVA depth distribution from the brain surface. Scale bars, 100 μm and 50 μm (insets). n = 5 mice/group; two-tailed unpaired Welch’s t test. L, Representative images showing anti-aquaporin 4 (AQP4) staining. Scale bar, 50 μm. M, Quantification of AQP4 coverage. n = 5 mice. N, Quantification of AQP4+ blood vessels. For m and n, n = 5 mice/group; two-tailed unpaired Welch’s t test. O, Brain sections were co-stained for anti-AQP4 (yellow) and anti-CD31 (cyan), and perpendicular lines to blood vessels were used to measure the perivascular space. Scale bars, 50 μm and 10 μm (insets). P, Representation of the perivascular space (PVS) in PBS (left) and CLO (right) treated mice. Q, Quantification of perivascular space. n = 4 mice treated with PBS, and 5 mice treated with CLO; two-tailed unpaired Welch’s t test.

R, One week after PBM depletion, mice received an i.c.m. injection of fluorescent beads (0.1 μm thick; 5 μl) and then were immediately placed on their side under the stereomicroscope for dynamic bead imaging at the MCA level. S, Representative images showing bead distribution over an hour at the proximal part of the MCA. Scale bar, 1 mm. T, Quantification of bead coverage at the MCA level over time. n = 4 mice treated with PBS, and 7 mice treated with CLO; repeated measures 2-way ANOVA with Geisser-Greenhouse correction. U, Representative images showing beads located at the MCA perivascular space in vivo (left) and ex vivo after perfusion with PBS and post-fixation in 4% PFA (right) from the same mouse. Scale bar, 1 mm. V, Representative images from extracted whole brain showing bead repartition at low (left image) and higher magnification (right image) and corresponding plot profile. The beads (green line) are located outside of the MCA (red line), at the perivascular level. Scale bar, 2 mm. W, Measure of the perivascular space (PVS) between in vivo and ex vivo from the same mice. n = 4 mice: Two-tailed paired t-test. X, Representative images showing ex vivo bead repartition at the MCA level in PBM-depleted mice and PBS-treated control mice, and corresponding quantification of bead coverage. Scale bar, 300 μm. n = 4 mice treated with PBS, and 5 mice treated with CLO; two-tailed unpaired Welch’s t-test. Y, Quantifications of total perivascular space (space between the two sides of the MCA where beads were found to accumulate) and MCA diameter (identified by the i.v. lectin injection). Z, Quantification of the functional space where beads were found to be accumulated. For y and z: n = 10 mice treated with PBS, and 9 mice treated with CLO; two-tailed unpaired Welch’s t-test. All data are presented as mean values +/- SEM. The illustration of mice in a, f and r are from Servier Medical Art, CC BY 3.0.
**Extended Data Fig. 5** PBM depletion has mild effect on mouse behavior and no effect on vital signs. One week after PBM depletion, mice underwent a battery of different behavioral tests. 

**a**, Cued fear conditioning: quantification of the percentage of time spent freezing during the three shocks the first day, and exposure to conditioned clues at one- and seven days after fear conditioning. 

**b**, Elevated plus maze: quantifications of the percentage of time spent in the open arm (left), the total time spent in the open arm (middle) and the total distance moved (right).

**c**, Open field test: quantification of the distance moved over an hour, the total distance moved, the time spent in the center of the box over an hour and the total time spent in the center of the box.

**d**, Forced swim test: quantification of the total floating time (left) and the latency to float (right). For **a-d**: n = 10 mice/group; two-tailed unpaired Welch’s t-test.

**e**, Three-chamber test: mice were first exposed to a mouse (S1) or an object (O), and then to a previously-exposed mouse (S1) or a new mouse (S2). Quantifications of the total sniffing time and the total time spent in the chamber for the two tests. n = 17 mice treated with PBS, and 13 mice treated with CLO; two-tailed unpaired Welch’s t-test. 

**f**, Vital signs: respiratory rate, heart rate, arterial pulsation and diameter were monitored. n = 5 mice/group; two-tailed unpaired Welch’s t-test. All data are presented as mean values ± SEM.
Extended Data Fig. 6 | See next page for caption.
Extended Data Fig. 6 | PBMs and extracellular matrix remodelling.

a, One week after CLO or PBS liposome injection, mice were perfused, brains were extracted, lateral choroid plexuses were removed, CD45-CD13+ and CD45-CD31+ cells were sorted and used for single-cell RNA sequencing. Nine different cell types were identified based on canonical markers.

b, Volcano plot corresponding to up- and down-regulated genes comparing fibroblasts in PBM-depleted mice and PBS-treated control mice, and corresponding GO Pathway analyses showing up- (left) and down-regulated (right) pathways.

c, Volcano plot corresponding to up- and down-regulated genes comparing pericytes in PBM-depleted mice and PBS-treated control mice, and corresponding GO Pathway analyses showing up- (left) and down-regulated (right) pathways.

d, Volcano plot corresponding to up- and down-regulated genes comparing capillary endothelial cells (cECs) in PBM-depleted mice and PBS-treated control mice, and corresponding GO Pathway analyses showing up- (left) and down-regulated (right) pathways.

For b–d, Volcano plots: F-test with adjusted degrees of freedom based on weights calculated per gene with a zero-inflation model and Benjamini-Hochberg adjusted P values; GO-pathways analyses: over-representation test.

e, Representative images showing cortical brain sections stained for anti-CD13 (mural cells, yellow) co-stained for anti-CD206 (magenta), anti-Laminin (cyan) and DAPI, and corresponding quantification. Scale bar, 100 μm; n = 5 mice/group; two-tailed unpaired Welch’s t-test.

f, Representative images showing cortical brain sections stained for anti-CD31 (endothelial cells) and corresponding quantification. Scale bar, 200 μm; n = 6 mice/group; two-tailed unpaired Welch’s t-test.

g, Representative images showing cortical brain sections of mice that were i.c.m. injected with OVA (magenta), stained for anti-αSMA (vascular smooth muscle cells, cyan) and co-stained for anti-CD31 (yellow), and corresponding quantification. Scale bar, 200 μm; n = 6 mice/group; two-tailed unpaired Welch’s t-test.

h, One week after CLO or PBS liposome injection, brain coronal sections were stained for anti-Laminin (cyan) and DAPI (blue). Scale bar, 2 mm.

i, High magnification images showing Laminin (cyan) in association with CD31+ blood vessels (red). Scale bar, 200 μm.

j, Representative Western blot images of Collagen-IV (160 kDa) and Ponceau S from isolated brain blood vessels one week after PBM depletion, and corresponding quantification. n = 5 mice/group; two-tailed unpaired Welch’s t-test.

k, Representative images showing Collagen-IV (cyan) deposition at both αSMA+ (arteries/arterioles; yellow) and αSMA- blood vessels. Scale bar, 200 μm.

l, Representative images from mouse cortex showing accumulation of Collagen-IV (cyan) in association with αSMA+ surface and penetrating large blood vessels (magenta), and corresponding quantifications. n = 6 mice/group; two-tailed unpaired Welch’s t-test.

m, High magnification images showing Laminin (cyan) in association with αSMA+ surface and penetrating large blood vessels (magenta), and corresponding quantifications. For m and n: Scale bar, 200 μm; n = 5 mice/group; two-tailed unpaired Welch’s t-test.

p, Representative images showing OVA distribution in whole brains and corresponding quantifications.

q, Representative images showing OVA coverage on brain coronal sections and corresponding quantifications. For p and q: Scale bars, 2 mm; n = 6 mice treated with PBS, 4 mice treated with CLO in saline group; 3 mice treated with PBS, and 5 mice treated with CLO in dobutamine group; two-way ANOVA with multiple comparisons. All data are presented as mean values +/- SEM.

The illustrations of mice in a and o are from Servier Medical Art, CC BY 3.0.
Extended Data Fig. 7 | See next page for caption.
Extended Data Fig. 7 | LYVE1+ PBMs drive CSF flow dynamics. a, Single-cell RNA sequencing was performed on whole brain samples. Seventeen cell types were identified based on canonical markers. b, Scanning electron microscopy image showing interactions between a PBM and a vascular smooth muscle cell (VSMC). Scale bars, 2 μm. c, Dot plot for Mrc1 (CD206), *H2-Ab1* (MHCII), Cd74, Cd163 and Lyve1 expression in each PBM cluster. d and e, Volcano plot and GO pathway analysis showing up- and down-regulated pathways in PBM cluster 2 versus other PBM clusters. Volcano plots: F-test with adjusted degrees of freedom based on weights calculated per gene with a zero-inflation model and Benjamini-Hochberg adjusted P values; GO-pathways analyses: over-representation test. f, Representative images suggesting interactions between LYVE1+ PBMs (magenta) and αSMA+ (yellow) VSMC. Scale bar, 200 μm and 30 μm (inset). g, Quantification of LYVE1+ cells associated or not with αSMA+ blood vessels. n = 5 mice. h, Mice received an i.v. injection of lectin and were perfused few minutes later. Whole brains were extracted, post-fixed with 4% PFA, and stained for anti-αSMA (green) and anti-LYVE1 (top panels) or anti-MHCII (bottom panels) (cyan). Scale bars: 1mm and 200 μm (insets). i, Characterization of PBM depletion in Lyve1Cre::Csf1r^fl/fl mice (Cre+) in brain coronal sections using CD206 staining (co-stained for DAPI) versus control littermates not expressing Cre (Csf1r^fl/fl; Cre-). Scale bar, 500 μm; n = 7 Cre- mice, and 3 Cre+ mice; two-tailed unpaired Welch’s t-test. All data are presented as mean values ± SE. The illustrations of mice (h, q) and the MRI scanner (q) are from Servier Medical Art, CC BY 3.0.
Extended Data Fig. 8 | See next page for caption.
Extended Data Fig. 8 | PBMs in normal aging. 

**a**, Experimental schematic: young adult (3-month-old) and aged (24-month-old) mice received an i.c.m. injection of OVA (45 kDa; 1mg/ml; 5 μl). Immediately after the injection, mice were placed on their side under the stereomicroscope for dynamic imaging. **b**, Representative images showing OVA distribution over time. Scale bar, 1 mm. **c**, Quantification of OVA signal fold increase over time in 3 m and 24 m old mice. n = 5 mice/group; repeated measures 2-way ANOVA with Geisser-Greenhouse correction. **d** and **e**, 3m and 24 m old mice received an i.c.m. injection of OVA. Mice were perfused one hour later. **d**, Representative images showing OVA distribution in whole brains in 3 m and 24 m old mice, and corresponding quantification. Scale bar, 5 mm. **e**, OVA coverage in coronal sections in 3 m and 24 m old mice, and corresponding quantification. Scale bar, 2 mm. For **d** and **e**: n = 3 m mice, and 4 24 m mice; two-tailed unpaired Welch’s t-test. **f**, 3m and 24m old mice received an i.c.m. injection of Texas Red (3 kDa; 1mg/ml; 5 μl), brains were harvested one hour later. Representative images of Texas Red coverage (red) and corresponding quantification. Scale bar, 2 mm. n = 5 mice/group; two-tailed unpaired Welch’s t-test.

**g**, Pie chart representation of the quantification of LYVE1+MHCII- versus LYVE1-MHCII+ PBMs by immunostaining. n = 5 mice/group; two-tailed unpaired Welch’s t-test. **h**, Representative flow cytometry plots from 3 m and 24 m old mice showing PBM subtypes, characterized by their expression of CD38 and MHCII. **i**–**k**, Quantification of **i**: CD206+ PBMs; **j**: MHCII+CD38- PBMs and **k**: MHCII-CD38+ PBMs. For **i**–**k**: n = 6 mice/group; two-tailed unpaired Welch’s t-test. **l**, 3m and 24m old mice received an i.c.m. injection of pHrodo particles (1 μm; 5 μl), which became fluorescent only after being phagocytosed. Scale bar, 2 mm. **m**, Representative confocal image showing pHrodo particles being phagocytosed by CD206+ PBMs. Scale bar, 50 μm. **n**, pHrodo-positive PBMs can also be detected by flow cytometry. **o**, Quantification of pHrodo+MHCII-CD38+ versus pHrodo+MHCII+CD38- PBMs in 3 m old mice. n = 6 mice; paired t-test. **p**, Representative images of 3 m and 24 m old mice showing pHrodo particle repartition at the MCA level, and corresponding quantification. Scale bar, 2 mm. **q**, Quantification of pHrodo+CD206+ PBMs in 3 m and 24 m old mice. For **p** and **q**: n = 6 mice/group; two-tailed unpaired Welch’s t-test. **r**, Quantification of pHrodo+MHCII-CD38+ versus pHrodo+MHCII+CD38- PBMs in 24 m old mice. n = 6 mice; paired t-test. **s**, 3 m and 24 m old mice received an i.c.m. injection of fluorescent beads (0.1 μm thick; 5 μl). Mice were perfused one hour later. Representative image from extracted whole brain showing bead repartition at low (left images) and higher magnification (insets, right images). Scale bars, 2 mm and 1 mm (insets). **t**, Quantification of brain bead coverage. **u**, Quantifications of total perivascular space (space between the two sides of the MCA where beads were found to accumulate). **v**, Quantification of MCA diameter (identified by the i.v. lectin injection). **w**, Quantification of the functional space where beads were found to be accumulated. For **t**–**w**: n = 5 mice/group; two-tailed unpaired Welch’s t-test. **x**, Brain coronal sections from 3 m and 24 m old mice were co-stained for anti-AQP4 (yellow) and anti-CD31 (cyan) to measure the perivascular space size. Scale bars, 50 μm and 10 μm (insets), and representation of the perivascular space (PVS) diameter in 3 m (middle) and 24 m (right) old mice. **y**, Quantification of perivascular space diameter. n = 5 mice/group; two-tailed unpaired Welch’s t-test. **z**, (Left) Brain coronal sections were stained for anti-Laminin (cyan) and DAPI (blue). Scale bar, 2 mm. (Right) High magnification images showing Laminin (cyan) in association with CD31+ blood vessels (red). Scale bar, 200 μm. All data are presented as mean values +/- SEM. The illustrations of mice in **a** and **d** are from Servier Medical Art, CC BY 3.0.
Extended Data Fig. 9 | M-CSF treatment in old mice. 

**a**, Violin plots showing expression of Csf1r mostly by PBMs, monocytes and microglia, as well as expression of Csf1, expressed mostly by endothelial, mural and microglial cells, from the mouse 5xFAD single-cell RNA sequencing dataset. 

**b** and **c**, Brain coronal sections of aged mice six hours after i.c.m. injection of artificial CSF (aCSF) or M-CSF (10 μg/ml; 5 μl) were stained for **b**: anti-Collagen-IV (left panels) or **c**: anti-Laminin (right panels) and co-stained for DAPI. Scale bars, 2 mm and 200 μm. 

**d**, Experimental schematic: 24 m old mice received an i.c.m. injection of M-CSF (or aCSF as a control), and mice received an i.c.m. injection of OVA to assess CSF flow 24 h later. 

**e**, Representative images showing OVA coverage at the MCA level, and corresponding quantification. Scale bar, 500 μm. n = 7 mice treated with aCSF, and 8 mice treated with M-CSF; two-tailed unpaired Welch’s t-test. 

**f**, Experimental schematic: 24 m old mice received an i.c.m. injection of M-CSF (or aCSF as a control), and MMP activity was assessed by fluorescence spectrometry 24 h later. 

**g**, Quantification of MMP activity. n = 8 mice/group; two-tailed unpaired Welch’s t-test. All data are presented as mean values +/- SEM. The illustrations of mice in **d** and **f** are from Servier Medical Art, CC BY 3.0.
Extended Data Fig. 10 | PBMs in an Alzheimer’s disease mouse model and in AD patients. **a** and **b**, One month after CLO or PBS liposome injection, 5xFAD mice received an i.c.m. injection of OVA, and brains were analyzed one hour later. **a**, Representative images and quantification of OVA distribution in whole brains. Scale bar, 2 mm. **b**, Representative images and quantification of OVA coverage on brain coronal sections. Scale bar, 2 mm. For **a** and **b**, n = 7 5xFAD mice treated with PBS, and 8 5xFAD mice treated with CLO; two-tailed unpaired Welch’s t-test. **c** and **d**, Quantiﬁcation of Aβ coverage in amygdala, cortex and hippocampus. n = 7 5xFAD mice treated with PBS, and 8 5xFAD mice treated with CLO; two-tailed unpaired Welch’s t-test. **e**, tSNE plot showing 35 different clusters on the 5xFAD mouse single-cell RNA sequencing dataset, based on expression of CD13, CD31 and CD45. **f**, Mcr1 expression in macrophage cluster allows PBM identification. **g**, Heatmap showing top 10 positively differentially expressed genes per cluster by adjusted p-value. **h**, GO Pathway analysis showing up- and down-regulated pathways in familial AD patients compared to controls. Over-representation test. **i**, Single-nuclei RNA sequencing on familial, pre-symptomatic, sporadic and non-AD patients. **j**, GO Pathway analysis showing up- and down-regulated pathways in familial AD patients compared to controls. Over-representation test. **k**, Gene expression levels of Ifngr1 and Ifngr2 from immune versus stromal cells from the mouse 5xFAD single-cell RNA sequencing dataset. **l** and **m**, Wild-type mice received an i.c.m. injection of artificial CSF (aCSF) or interferon gamma (IFNγ, 20 ng/ml; 1 μl). The same mice received an i.c.m. injection of OVA (1mg/ml; 5 μl) 3 h later and brains were harvested one hour later. **l**, Representative images and quantification of OVA distribution in whole brains. **m**, Representative images showing OVA coverage on brain coronal sections and corresponding quantification. For **l** and **m**, n = 5 mice/group; two-tailed unpaired Welch’s t-test. **n**, Proposed model that recapitulates the findings. All data are presented as mean values +/- SEM.
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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection
The following softwares were used to collect the data in this study:
- LAS X Version: 4.3.0.24308, Leica Microsystems CMS GmbH
- Olympus V200 ASW 3.3 (Build 24382)
- Kaluza Analysis Software v1.0
- Paravision 360.2.0.1pl.1

Data analysis
The following softwares were used to analyze the data in this study:
- FIJI image processing software [NIH] - v2.3.0/1.53f
- Prism v9.2.0 (GraphPad Software, Inc)
- FastQC v0.11.5
- Salmon v0.8.2
- R v3.5.0
- MATLAB 9.7
- Cytobank 7.2
- Bioconductor DESeq2 v3.5
- Custom code used for single-cell and single-nucleus RNA-seq analysis is available from the corresponding authors upon reasonable request.

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- A list of figures that have associated raw data
- A description of any restrictions on data availability

Source data files depicting the quantification values mentioned in the text or plotted in graphs shown in Figs. 1-5 and Extended Data Figs. 1-10, are available in the online version of this paper at http://www.nature.com/nature. RNA-seq data sets have been deposited online in the Gene Expression Omnibus (GEO database) under the accession numbers GSE188283, GSE188284, GSE188285. Custom code used to analyze the RNA sequencing data is freely available at https://doi.org/10.5281/zenodo.7047054. All other data generated in this study are available online in a GraphPad Prism file. To access the data from the Dian brain bank, special request must be made using this URL: https://dian.wustl.edu/our-research/for-investigators/.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- [X] Life sciences
- [ ] Behavioural & social sciences
- [ ] Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

**Sample size**
Sample sizes were chosen on the basis of standard power calculations (with \( \alpha = 0.05 \) and power of 0.8) performed for similar experiments that were previously published (Louveau et al., Nature Neuroscience 2018; Da Mesquita et al., Nature 2021). In general, statistical methods were not used to re-calculate or predetermine sample sizes.

**Data exclusions**
The ROUT test was used to identify and discard potential outliers. No exclusion criteria were pre-established. Mice that died during the Magnetic Resonance Imaging (MRI) were excluded from the study (2 mice).

**Replication**
Number of reliable reproductions of each experimental finding is stated in each Figure legend. Unless stated otherwise, main experimental findings were replicated at least once. Experimental replication was not attempted for negative data.

**Randomization**
Reported in Methods section, Statistical analysis and reproducibility subsection: Animals from different cages, but within the same experimental group, were selected to assure randomization. Mice from the same cage received different treatments (for example, in a cage of 5 mice, 2 mice received PBS-loaded liposomes and 3 mice received CLO-loaded liposomes). Treatment was given in a blind manner and could be identified by a corresponding ear tag (treatment A: left ear was tagged; treatment B: right ear was tagged).

**Blinding**
Reported in Methods section, Statistical analysis and reproducibility subsection: Experimenters were blinded to the identity of experimental groups from the time of experimental treatment until the end of data collection and analysis for all the independent experiments. One exception was the experiment regarding CSF flow dynamics between young (3-month-old) and aged (24-month-old) mice, which are distinguishable by their weight/fur.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

| Materials & experimental systems | Methods |
|----------------------------------|---------|
| n/a | n/a |
| [X] | [X] |
| Antibodies | Involved in the study |
| [ ] | [ ] |
| [X] | [X] |
| Eukaryotic cell lines | ChiP-seq |
| [ ] | [ ] |
| [X] | [X] |
| Palaeontology | Flow cytometry |
| [ ] | [ ] |
| [X] | [X] |
| Animals and other organisms | MRI-based neuroimaging |
| [ ] | [ ] |
| [X] | [ ] |
| Human research participants | |
| [ ] | |
| [X] | |
| Clinical data | |

**Antibodies**

Antibodies used

The following primary antibodies were used for IHC:
Validation

Each antibody was validated for the species (mouse) and application (immunohistochemistry, cell sorting) by the correspondent manufacturer. The usage was described in full detail the methods section of the manuscript.

Immunohistochemistry:

- Tissue was rinsed in PBS and incubated with appropriate dilutions of primary antibodies (from the list described in the previous section) in PBS 0.5% Triton-X 100 overnight at 4°C. Whole mounts or tissue sections were then washed 3 times for 5 min at RT in PBS 0.5% Triton-X 100 followed by incubation with the appropriate secondary antibodies (from the list described in the previous section) for 2 hours at RT in PBS 0.5% Triton-X 100.

- Sorting of whole brain hemisphere myeloid (microglia/macrophage), blood endothelial and mural cells or of brain cortical myeloid cells.

- Briefly, after tissue digestion, the cell pellets were washed, resuspended in ice-cold FACS buffer, preincubated for 10 min at 4°C with Fc-receptor blocking solution (anti-CD16/32, 101302, Biologic, 1:200 in FACS buffer) and stained for extracellular markers with a mix of primary antibodies from the list described in the previous section, for 25 minutes at 4°C.

Animals and other organisms

Policy information about studies involving animals: ARRIVE guidelines recommended for reporting animal research

Laboratory animals

- Mice were bred in house or obtained from the Jackson Laboratory or provided by the National Institutes of Health/National Institute on Aging (24-month-old mice). Mice were housed in 12-hour light-dark cycle in a temperature- and humidity-controlled environment with water and food ad libitum. Mice were housed at least one week at the animal facility before any experimentation. Both males and females were used in this study. The following mouse strains were used in this study: C57BL/6j (WT; JAX 000664), C57BL/6-Tg [UbcGFp; JAX 004353], hemizygous B6SJL-Tg [APPSwFlon, PSEN1* M146L, 1286V, 6999Vas; Mmja] (b6.A129P2-Lv45tm12.LtEGFPcre/J; JAX 0012601), Csf1rfl/fl [b6.CgCsfr1tm122wos/J; JAX 021212], Aldh1l1Cre/Ert2[b6; JAX 031008].

Wild animals

- The study did not involve wild animals.

Field-collected samples

- The study did not involve samples collected from the field.

Ethics oversight

- All experiments were approved by the Institutional Animal Care and Use Committee of the University of the Missouri and the Institutional Animal Care and Use Committee of the Washington University in Saint-Louis.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Human research participants

Policy information about studies involving human research participants

Population characteristics

- Covariate-relevant population characteristics are publicly available in Da Mesquita et al., Nature 2021, Supplementary Table 3.

Recruitment

- The Neuropathology Cores of the Charles F. and Joanne Knight Alzheimer’s Disease Research Center (Knight-ADRC) and the Dominantly Inherited Alzheimer Network (DIAN) provided the parietal lobe tissue of postmortem brains for each sample. These samples were obtained with informed consent for research use and were approved by the review board of Washington University in St. Louis. AD neuropathological changes were assessed according to the criteria of the National Institute on Aging-Alzheimer’s Association (NIA-AA). Their demographic, clinical severity, and neuropathological information are presented in Supplementary Table 3.

Ethics oversight

- The brain samples used in the single-nucleus RNA-seq experiments were obtained with informed consent for research use and were approved by the review board of Washington University in St. Louis.
Flow Cytometry

Plots

- Confirm that:
  - The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
  - The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
  - All plots are contour plots with outliers or pseudocolor plots.
  - A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation
Mice received a lethal intraperitoneal injection of euthasol (10% v/v in saline, 250 l), and transcardially perfused with PBS containing 10U/ml heparin. Lateral choroidplexuses were removed from half- or whole brains. Brains were then digested for 40 min at 37°C with 1.4U/ml of Collagenase-VIII and 35U/ml of DNAse I in DMEM. The cell pellets were washed and resuspended in FACS buffer and stained with the following antibodies (1:200 dilution; ebioscience): rat anti-mouse Ly6G (BV510), rat anti-mouse XCR1 (BV421), rat anti-mouse CD24 (Pacific Blue), rat anti-mouse Ly6G (BVs61), rat anti-mouse CD45 (BV750), rat anti-mouse CD19 (BVs615), rat anti-mouse CD11b (BVs663), rat anti mouse TCRb (BV805), rat anti mouse CD4 (BVs395), rat anti mouse F4/80 (BVs605), rat anti-mouse CD64 (PerCP-Cy5.5), rat anti-mouse CD38 (A647 or Pacific Blue), rat anti-mouse MHCII (BVs650). Cell viability was determined using DAPI staining. Fluorescence data were acquired using Cytek Aurora spectral flow cytometer (Cytek) then analyzed using FlowJo Software (Tree Star, v5.0). For one experiment, mice received an i.c.m. injection of phpHrodo particles (Life Technologies, P35360), that were detected by A647.

Instrument
- Aurora™ Flow Cytometer (Cytek)

Software
- FlowJo software v10.8.1 (Tree Star, Inc.)

Cell population abundance
For each individual experiment, single-cell suspensions were incubated with viability dyes and aliquots from the unstained single-cell suspensions were incubated with ViaStain™ AOPI Staining Solution (CS2-0106, Nexcelom Bioscience)

Gating strategy
Flow cytometry and FACS using brain tissue preparations:
Briefly, singletons were gated using the height, area and the pulse width of the forward and side scatters and then viable cells were selected as being negative for SYTOX or DAPI. Whole brain hemisphere myeloid (microglia/macrophage) cells were identified and sorted as DAPI−CD45−CD11b−Ly6G−. Blood endothelial and mural cells were identified and sorted as DAPI−CD45−CD11b−CD31+ and DAPI−CD45−CD11b−CD13+CD31−, respectively. Brain cortical myeloid cells were identified and sorted as SYTOX−Ly6G−CD45−CD11b−.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.

Magnetic resonance imaging

Experimental design

Design type
Evaluation of CSF flow using i.c.m. Dotarem injection and tracing over time

Design specifications
The total acquisition time was about 1 h per mouse (4ms4sec x 12 sequences per mouse).

Behavioral performance measures
None

Acquisition

Imaging type(s)
Contrast-enhanced MRI

Field strength
9.4 Tesla

Sequence & imaging parameters
Series of post-contrast T1 FLASH-3D weighted images were taken through the head with the following parameters: repetition time = 30 ms; echo time = 8 ms; number of echo images = 1; number of averages = 1; number of repetitions = 12; scan time = 272640 ms per sequence (4ms4sec); flip angle = 20, FOV = 160 x 160 x 80 mm with a 128 x 128 x 64 matrix; spatial resolution = 125 x 125 x 125 μm (8 pixels per mm; voxel size = 0.125 mm3), number of slices = 64; receiving coil 4 elements RF ARR 400 1H M.

Area of acquisition
Mouse head

Diffusion MRI
- Used
  - Not used
### Preprocessing

| Description               | Details                                    |
|---------------------------|--------------------------------------------|
| Preprocessing software    | Paravision 360.2.0.pl.1                     |
| Normalization             | Each mouse was its own control             |
| Normalization template    | Each mouse was its own control (diffusion of tracer over time) |
| Noise and artifact removal| No noise or artifact removal                |
| Volume censoring          | No volume censoring                        |

### Statistical modeling & inference

| Description               | Details                                    |
|---------------------------|--------------------------------------------|
| Model type and settings   | Tracer diffusion overtime per mouse (univariate) |
| Effect(s) tested          | Repeated measure ANOVA                     |
| Specify type of analysis: | ☑ Whole brain                             |
|                           | ☑ ROI-based                                |
|                           | ☑ Both                                     |
| Anatomical location(s)    | Whole brain, middle cerebral artery territory |
| Statistic type for inference | [Specify voxel-wise or cluster-wise and report all relevant parameters for cluster-wise methods.](Eklund et al., 2016) |
| Correction                | Geisser-Greenhouse correction              |

### Models & analysis

| Description               | Details                                    |
|---------------------------|--------------------------------------------|
| n/a                       | Involved in the study                      |
| ☑ ☑                  | Functional and/or effective connectivity    |
| ☑ ☑                  | Graph analysis                             |
| ☑ ☑                  | Multivariate modeling or predictive analysis |