Origin, fate and dynamics of macrophages at central nervous system interfaces

Tobias Goldmann1,14, Peter Wieghofer1,2,14, Marta Joana Costa Jordão1,2,14, Fabiola Prutek1, Nora Hagemeier1, Kathrin Frenzel1,2, Lukas Amann1,2, Ori Staszewski1, Katrin Kierdorf1, Martin Krueger3, Giuseppe Locatelli4, Hannah Hochgerner5, Robert Zeiser6,7, Slava Epelman8, Frederic Geissmann9, Josef Priller10, Fabio M V Rossi11, Ingo Bechmann3, Martin Kerschensteiner4,12, Sten Linnarsson5, Steffen Jung13 & Marco Prinz1,7

Perivascular, subdural meningeal and choroid plexus macrophages are non-parenchymal macrophages that mediate immune responses at brain boundaries. Although the origin of parenchymal microglia has recently been elucidated, much less is known about the precursors, the underlying transcriptional program and the dynamics of the other macrophages in the central nervous system (CNS). It was assumed that they have a high turnover from blood-borne monocytes. However, using parabiosis and fate-mapping approaches in mice, we found that CNS macrophages arose from hematopoietic precursors during embryonic development and established stable populations, with the notable exception of choroid plexus macrophages, which had dual origins and a shorter life span. The generation of CNS macrophages relied on the transcription factor PU.1, whereas the MYB, BATF3 and NR4A1 transcription factors were not required.

Under steady-state conditions, the CNS hosts a heterogeneous population of myeloid cells, including parenchymal microglia and non-parenchymal perivascular, subdural meningeal and choroid plexus macrophages1–3. The latter myeloid cells are critical effectors and regulators of immune responses at CNS borders during virtually all neuroinflammatory, neurodegenerative and neuro-oncological diseases.

Unlike microglia, which are derived from early yolk sac precursors before birth4–7, all other CNS macrophages found in the perivascular (Virchow-Robin) spaces, subdural meninges and choroid plexus were believed to originate from short-lived blood monocytes after birth that are quickly replaced by bone marrow (BM)-derived cells8,8. These assumptions were made on the basis of cell transplantation experiments in rodents starting in the 1980s (refs. 9,10). Subsequently, chemotherapeutical conditioning also suggested that perivascular macrophages were present in the CNS parenchyma after BM transplantation11,12. However, all of these studies used either irradiation or chemotherapy as conditioning regimens, thereby inducing an artificial influx of injected BM-derived cells, likely as a result of changes of the blood-brain barrier and local induction of chemoattractants in the host CNS13–15.

Thus, it has been believed for decades that microglia and non-parenchymal macrophages in the subdural meninges, perivascular spaces and choroid plexus represent two ontogenetically and transcriptionally distinct myeloid populations. By combining large-scale single-cell RNA-sequencing with multiple approaches of fate-mapping, parabiosis and in vivo imaging, we challenged the view that non-parenchymal macrophages are BM derived and provide new insights into the transcriptional networks, ancestry and turnover of these macrophages at CNS boundaries. We also found that non-parenchymal CNS macrophages are closely related to microglia, but still represent a distinct specialized population of tissue macrophages.

RESULTS
Gene expression profiling of CNS macrophages
Myeloid cells in the brain are a diverse group of cells localized at strategic places of the CNS. The parenchyma is populated with microglia, whereas tissue borders host lba-1+ macrophages that can be found in the subdural meninges (mMΦ), perivascular spaces (pvMΦ) and choroid plexus (cpMΦ) (Fig. 1a).

CNS macrophages have historically been classified on the basis of their distinct anatomical localization, morphology and expression of selected molecular markers15. In detail, pvMΦ have been described to express CD206, a receptor on non-parenchymal macrophages in the CNS and to be sandwiched between the laminin-positive endothelial and glial basement membranes16. In contrast, subdural mMΦ are

1Institute of Neuropathology, Freiburg University Medical Centre, Freiburg, Germany. 2Faculty of Biology, University of Freiburg, Freiburg, Germany. 3Institute of Anatomy, University of Leipzig, Leipzig, Germany. 4Institut für Klinische Neuroimmunologie, Ludwig-Maximilians Universität München, Munich, Germany. 5Division of Molecular Neurobiology, Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm, Sweden. 6Department of Hematology and Oncology, Freiburg University Medical Centre, Freiburg, Germany. 7BIOSST Centre for Biological Signaling Studies, University of Freiburg, Freiburg, Germany. 8Peter Munk Cardiac Centre, University Health Network Toronto, Ontario, Canada. 9Centre for Molecular and Cellular Biology of Inflammation, King’s College London, London, UK. 10Department of Neuropsychiatry and Laboratory of Molecular Psychiatry, Charité – Universitätsmedizin Berlin; Cluster of Excellence NeuroCure, DZNE & BBI, Berlin, Germany. 11The Biomedical Research Centre, Faculty of Medicine, University of British Columbia, Vancouver, British Columbia, Canada. 12Munich Cluster for Systems Neurology (SyNergy), Munich, Germany. 13Department of Immunology, The Weizmann Institute of Science, Rehovot, Israel. 14These authors contributed equally to this work. Correspondence should be addressed to M.P. (marco.prinz@uniklinik-freiburg.de).
localized in close vicinity to ER-TR7+ fibroblast-like cells that line the meninges and meningeal vasculature. Finally, cpMΦ are found exclusively in the stroma and epithelial layer of the choroid plexus. Using confocal microscopy and subsequent three-dimensional reconstruction of brain tissue from mice in which one allele of the gene encoding the cytokine receptor CX3CR1 contains the gene encoding GFP (Cx3cr1<sup>GFP</sup>+/−), we were able to accurately identify Iba-1<sup>+</sup>CX3CR1<sup>+</sup> pvMΦ on the basis of their close proximity to the abluminal side of endothelial cells (CD31<sup>−</sup>) between the endothelial and the parenchymal basement membrane (laminin<sup>+</sup>), the perivascular space (Fig. 1b). In the subdural meninges, we localized Iba-1<sup>+</sup>CX3CR1<sup>+</sup> mMΦ by their close proximity to fibroblasts expressing ER-TR7+ (Fig. 1c).

We then used an unbiased, surface marker-free approach to thoroughly molecularly characterize cortical microglia and cortical pvMΦ. For that purpose, we prepared a whole-cortex cell suspension devoid of meninges and choroid plexus. We then performed unbiased quantitative single-cell RNA sequencing of microglia, pvMΦ, their proposed precursors, the monocytes and peritoneal macrophages (ptMΦ) (Fig. 1d and Supplementary Figs. 1 and 2). Individual RNA molecules were counted using unique molecular identifiers (UMIs) as described previously<sup>18</sup>, which greatly reduces PCR amplification bias. Dimensionality reduction using t-distributed stochastic network embedding (t-SNE)<sup>19</sup> revealed that pvMΦ and microglia were transcriptionally closely related, whereas monocytes and ptMΦ had a distinct RNA profile. All myeloid populations expressed the genes encoding the markers CX3CR1, CSF-1R and Iba-1 (Fig. 1e), but pvMΦ were distinguishable from microglia on the basis of their expression of Mrcl (encoding CD45) and Cd36 (Fig. 1f). Microglia expressed higher levels of P2ry12 and Hexb than pvMΦ (Fig. 1g), whereas monocytes exclusively expressed Cdc20 (Supplementary Fig. 1). Notably, Ptprc (encoding CD45) mRNA transcript expression was higher in pvMΦ than in microglia (Fig. 1e), which could be confirmed at the protein level for all non-parenchymal macrophages in situ, but not for microglia (Fig. 1h).

Furthermore, the detection of Cd11b protein on Cx3cr1<sup>GFP</sup>+/− pvMΦ located in the perivascular space (laminin<sup>+</sup>), but not on parenchymal microglia, is consistent with our findings on RNA level (Fig. 1i). Flow cytometry-based separation of Cd45<sup>−</sup>Cd11b<sup>−</sup>Cd206<sup>−</sup> pvMΦ and Cd45<sup>−</sup>Cd11b<sup>+</sup>Cd206<sup>+</sup> microglia from mouse cortex that was free of meninges and choroid plexus enabled us to confirm the results obtained from the single-cell RNA sequencing on the protein level in a quantitative manner (Fig. 1j,k and Supplementary Fig. 2).

Taken together, these data show a close transcriptional relationship between non-parenchymal macrophages such as pvMΦ and microglia, albeit with some cell type–specific differences.
Prenatal origin of CNS macrophages

The current concept that macrophages at CNS borders are derived from blood-borne myeloid cells during adulthood largely depends on previous work on BM chimeras using irradiation of the recipient\(^{20,21}\), a finding that we were able to confirm by transplanting BM from Acta1−/−GFP mice, which ubiquitously express GFP in all cells of the body, into irradiated hosts, which led to a vigorous engraftment of donor-derived GFP+Iba-1+ macrophages in the subdural meninges, perivascular spaces and choroid plexus, whereas microglial exchange was limited (Supplementary Fig. 3).

However, in several organs, resident macrophages arise from embryonic precursors in the yolk sac or fetal liver that seed the tissue before birth\(^{13,15}\). Consistent with these findings, we observed Cx3cr1\(^{GFP/WT}\) macrophages in the subdural meninges at embryonic day 9.5 (E9.5) and shortly thereafter in the choroid plexus and the perivascular spaces (Fig. 2), suggesting prenatal seeding of tissue macrophages in these compartments. To test whether embryonic progenitors indeed contribute to the pool of CNS macrophages, we adapted a Cx3cr1\(^{CreER}\) mouse system that allows fate-mapping studies by tamoxifen-induced recombination in Cx3cr1-expressing cells at defined time points\(^{25,26}\). Timed pregnancies were set up to generate Cx3cr1\(^{CreER}\) Rosa26-YFP embryos that undergo recombination and subsequently express YFP under the control of the Rosa26 promoter after a single intraperitoneal injection of tamoxifen in pregnant wild-type mice at E9.0 to pulse Cx3cr1-expressing progenitor cells in the embryo (Supplementary Fig. 3a). This approach induced irreversible expression of YFP in Cx3cr1-expressing cells and their progeny. At E16.0, 55.4 ± 3.3% Iba-1+ mMΦ, 51.3 ± 11.5% Iba-1+ cpMΦ and 36.2 ± 7.5% Iba-1+ microglia expressed YFP, indicating robust labeling efficiency of the Cx3cr1\(^{CreER}\) progenitors (Fig. 3a,b). Although YFP+ pvMΦ could be recognized at this embryonic stage, they were too sparse to allow a reliable quantification in Cx3cr1\(^{CreER}\) Rosa26-YFP embryos. Not only microglia, but also pvMΦ and mMΦ, retained the YFP label in 6-week-old mice, demonstrating that embryonic labeling persisted into adulthood (Fig. 3c,d). In contrast, cpMΦ lost their YFP label over time, indicating that embryo-derived cells were replaced in the choroid plexus. Expression of YFP in pvMΦ in the perivascular space was confirmed by immunoelectron microscopy (Fig. 3e).

Several transcription factors have been shown to be important for lineage commitment in myeloid cells\(^{27,28}\). To determine which transcription factors are required for non-parenchymal CNS MΦ development, we investigated the presence of CNS macrophages in mutant embryos lacking Sfpi1 (encoding PU.1), Irf8, Myb and Batf3 (Fig. 3f–h and Supplementary Fig. 4a). Mice lacking PU.1 were devoid of any pvMΦ, mMΦ and cpMΦ. Notably, we found a reduction in the numbers of mMΦ, but not cpMΦ in Irf8\(^{−/−}\) mice, whereas the absence of the master transcription factor for stem cell development in the BM, MYB, did not impair mMΦ and cpMΦ development, similar to its redundant role for yolk sac-derived macrophages such as microglia\(^{8}\). Batf3 deficiency did not impair the development of any of the investigated macrophage populations. In summary, our data suggest mMΦ, pvMΦ, cpMΦ and microglia are all of prenatal origin, either from the yolk sac and/or the fetal liver, and largely depend on similar transcription factors for proper development.

Maintenance of pvMΦ, mMΦ and cpMΦ in adulthood

Once established in the CNS, microglia persist throughout the entire life of the organism without any substantial input from circulating blood cells as a result of their longevity and their capacity for
self-renewal\textsuperscript{13,29}. Thus, we sought to use this unique feature of microglia to compare their exchange kinetics with mMΦ, pvMΦ, cpMΦ and blood cells, such as monocytes. We treated adult Cx3cr1\textsuperscript{CreER} Rosa26-YFP mice with tamoxifen and determined mMΦ, pvMΦ and cpMΦ labeling at several time points after application (Supplementary Fig. 4a). Flow cytometry confirmed the flow cytometry data and revealed a high percentage of Iba-1–labeled mMΦ, pvMΦ and cpMΦ that coexpressed YFP (Fig. 4b). Quantitative examination on histological slices showed constant high expression of the reporter gene in mMΦ and pvMΦ, comparable to what we observed in microglia (Fig. 3d). These data indicate that mMΦ and pvMΦ are stable populations that do not undergo substantial exchange with blood cells over 46 weeks. In contrast, YFP-labeling progressively dropped in Iba-1\textsuperscript{+} cpMΦ, suggesting a slow continuous exchange with blood cells. As a result of their transient nature Cx3cr1-targeted Ly6C\textsuperscript{hi} monocytes in the blood lost their YFP reporter very rapidly and were quickly replaced by the non-targeted progeny\textsuperscript{25,26,30}. Confocal analysis further confirmed the long-term persistence of pvMΦ in perivascular spaces of cerebral vessels (limited by laminin\textsuperscript{*} basement membranes) at 8 weeks after tamoxifen treatment (Fig. 4c,d). At this time point, microglia were still labeled, but blood monocytes were not (Fig. 4e). Notably, we detected YFP-expressing mMΦ and cpMΦ by immunoelectron microscopy 30 weeks after recombination (Fig. 4f). Labeled cpMΦ were found in close proximity to the microvilli of the choroid plexus epithelium, indicating that these cells represent Kolmer’s epiplexus cells.

To further analyze whether the presence of these long-lived myeloid cells in distinct CNS microenvironments shapes their dynamic behavior, we imaged fluorescently labeled mMΦ, pvMΦ and microglia of living Cx3cr1\textsuperscript{CreER} Rosa26-Tomato mice at 8 weeks after tamoxifen treatment by confocal and \textit{in vivo} two-photon (2p) microscopy. We found Tomato\textsuperscript{*} cells in the subdural meninges, perivascular space and choroid plexus that expressed the markers F4/80 and CD206, indicating their macrophage nature (Fig. 5a and Supplementary Fig. 4b). 2p time-lapse imaging revealed that mMΦ, pvMΦ and microglia, which are all embryo derived, can be differentiated \textit{in vivo} not only on the basis of their anatomical location, but also of their characteristic morphology and distinct dynamic behavior (Fig. 5b–e and Supplementary Videos 1 and 2). Consistent with previous
reports, microglial cells showed a ramified morphology with highly dynamic processes and a rather stationary cell body. In contrast, mM often presented with a more ameboid morphology, and about a third of these cells (62 of 172 cells) moved over time (Fig. 5c, 5f). These cell types could be further differentiated from pvM, which followed the blood vessel outline and rarely displaced their cell body (5 of 44 cells), but constantly extended and retracted their protrusions along the blood vessel wall.

To evaluate a possible contribution of blood cells to the pool of macrophages at CNS boundaries under homeostatic conditions, we needed a method for tracking circulating cells without affecting the CNS environment or resident cells. Thus, we induced peripheral blood
chimerism by surgically joining two syngeneic mice, one of which ubiquitously expressed GFP (Acta1-GFP). These parabiotic mice established a rich anastomotic circulation, which quickly led to efficient blood chimerism in peripheral Ly6C<sup>hi</sup> and Ly6C<sup>lo</sup> monocytes (Fig. 6a)<sup>33</sup>. However, there was no detectable circulatory exchange of mMΦ and pvMΦ after 5 months of parabiosis, whereas donor-derived blood cells contributed to the cpMΦ population (Fig. 6a). Furthermore, low gene recombination of mMΦ and pvMΦ in Flt3<sup>Cre</sup> Rosa26-YFP animals suggested that their development occurred largely independently of Flt3<sup>+</sup> multipotent hematopoietic precursors in the BM compared with the high percentage of YFP<sup>+</sup> myeloid cells in the circulation (Fig. 6b). Again, only cpMΦ displayed a higher recombination of this gene, suggesting a role for hematopoietic stem cells in the development of cpMΦ. Furthermore, we found that neither Ly6C<sup>hi</sup> nor Ly6C<sup>lo</sup> monocytes were essential for mMΦ and pvMΦ development, as mice lacking Ccr2 or Nr4a1 presented normal proportions of these macrophages subsets (Fig. 6c). Only cpMΦ were reduced in Ccr2-deficient mice, indicating that Ly6C<sup>lo</sup> monocytes contribute to their homeostasis. In sum, these data clearly indicate that non-parenchymal MΦ, such as mMΦ and pvMΦ, share their longevity with microglia without any input from blood monocytes (Supplementary Fig. 3). Only cpMΦ are exceptional, which, because of their dual origin, have a partial input from the circulation and a shorter turnover.

**DISCUSSION**

Our results describe the molecular signatures of tissue macrophages at CNS boundaries, their developmental pathways and the key mechanisms that ensure their homeostasis. By using large-scale single-cell RNA sequencing, we were able to dissect the individual transcriptional profiles of cortical pvMΦ and parenchymal myeloid cells (microglia) in the CNS and found a close relationship between these cells that was not shared by circulating monocytes or ptMΦ. Our unbiased...
high-throughput methodology allowed us to define extensive functional specialization between cell classes. In fact, we found that the marker Mrc1 was enriched in macrophages at CNS interfaces, whereas P2ry12 was expressed only by microglia, which confirms previous data that used expression profiling of several thousands of cells in the CNS. However, our finding that mMΦ, pvMΦ and cpMΦ have only limited relation to circulating myeloid cells represents a major conceptual change in the field. Since the 1980s, it has been assumed,...

Figure 6 Turnover of CNS macrophages. (a) Immunofluorescence microscopy (left) showing negligible exchange of cpMΦ and no contribution to pvMΦ, mMΦ and microglia in a wild-type parabiont after 5 months of parabiosis with an Acta1-GFP mouse. *, single-positive cells; arrows, double-positive cells; arrowheads, microglia. Scale bars, 25 µm. Right, quantification of results at left (left plot), and blood chimeraism for (CD45+ CD11b+ CD115+) Ly6C(lo) and Ly6C(hi) monocytes (far right). (b) Immunofluorescence microscopy (left) showing little Flt3 expression as a marker of definitive hematopoiesis in pvMΦ, mMΦ and cpMΦ in adult Flt3Cre+ Rosa26-YFP mice. Scale bars, 25 µm. Right, quantification of results at left (left plot), and recombination efficiency of circulating CD11b+ Gr-1+ CD3+ CD19- inflammatory monocytes (Gr-1+) and CD11b+ Gr-1+ CD3+ CD19- resident monocytes (Gr-1+), assessed by flow cytometry. (c) Iba-1 immunohistochemistry (brown) showing the localization and presence of pvMΦ, mMΦ and cpMΦ in adult wild-type (WT), Ccr2−/− and Nr4a1−/− mice (left), and quantification of results at left (right). Ccr2−/− mice (meninges: 3 months WT = 7 mice, 3 months KO = 6, unpaired t test P = 0.1795; 12 months WT = 5, 12 months KO = 5, unpaired t test P = 0.1354; perivascular space: 3 months WT = 7 mice, 3 months KO = 7, unpaired t test P = 0.9613; 12 months WT = 5, 12 months KO = 5, Mann-Whitney test P = 0.4206; choroid plexus: 3 months WT = 7 mice, 3 months KO = 7, Mann-Whitney test *P = 0.0379; 12 months WT = 5, 12 months KO = 5, unpaired t test ***P = 0.0005); Nr4a1−/− mice (meninges: WT = 7 mice, KO = 8, unpaired t test P = 0.7282; perivascular space: WT = 8 mice, KO = 8, unpaired t test P = 0.1566; choroid plexus: WT = 8 mice, KO = 8, unpaired t test P = 0.9658). NS, not significant (P > 0.05). Each symbol (a-c, right) represents an individual mouse; small horizontal lines indicate the mean ± s.e.m. Data are from one independent experiment with three mice (a, left images), four mice (a, far right) or n = 4 mice (mMΦ, pvMΦ and microglia) or n = 6 mice (cpMΦ) with at least three tissue sections (a, left plot), two independent experiments with three mice (b, left images), three mice (b, far right) or n = 6 mice per cell population (b, left plot) or two independent experiments with five mice per genotype (c, left), or four (wild-type and Ccr2−/−) or three (wild-type and Nr4a1−/−) independent experiments with at least three tissue sections (c, right).
sentinel T cells are thought to enter the cerebrospinal fluid through the choroid plexus during incipient stages of MS and EAE, and then travel to the subarachnoidal space, where they are reactivated by local antigen-presenting cells (APCs) presenting cognate antigen. These findings all argue for the dual role of the choroid plexus as an immune cell–modulatory site, as well as an entry gate for peripheral immune cells to the CNS during neuroinflammation.

Strategically positioned at the CNS barriers, mMF, pvMF and cpMF might modulate immune cell entry and phenotype. The myeloid cells in the CNS-adjoining tissues have been implicated in various immunopathological processes, including antigen presentation to circulating lymphocytes. As presumed guardians of tissue homeostasis, the CNS-surrounding myeloid cell network appears to be crucially involved in the development, progression and resolution of neuroinflammatory, neurodegenerative and neurooncological diseases. Our results will help to unravel mMF, pvMF and cpMF function in vivo in more detail and to identify new means to manipulate these cells for the treatment of neural diseases.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**ACKNOWLEDGMENTS**

We thank M. Oberle, M. Ditter and T. el Gaz for excellent technical assistance and T. Leng Tay for critical reading of the manuscript. Supported by the DFG (SFB 992, SFB 1160, PR 3778/1-1, Reinhart Koselleck Grant for M.P.; SFB 1160, E872/3-1 for R.Z.; and FOR1336 for I.B., J.P., M.P. and S.J.), the Fritz-Thyssen Foundation (M.P.), the European Union’s Seventh Framework Program FP7 under Grant agreement 607962 (nEUROinflammation for M.P.), the Gemeinnützige Hertie Foundation (GHST for M.P.), the Sobek Foundation (M.P.) and the BMBF-funded Competence Network on Multiple Sclerosis (KKNMS for M.P. and M. Kerschensteiner).

**AUTHOR CONTRIBUTIONS**

T.G., P.W., M.J.C.I., P.F., N.H., K.F., O.S., K.K., L.A., M. Krueger, G.L. and H.H. conducted the experiments and analyzed the data. R.Z., S.E., F.G., J.P., F.M.V., I.B., S.I., M. Kerschensteiner and S.J. analyzed the data, contributed to the in vivo studies and provided mice or reagents. T.G. and M.P. supervised the project and wrote the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

Reprints and permissions information is available online at [http://www.nature.com/reprints/index.html](http://www.nature.com/reprints/index.html).


Identification of a unique TGF-β ATP mediates rapid microglial response to local brain injury.
Fluorescence microscopy and cell quantifications. After transcardial perfusion with PBS, brains were fixed for 24 h in 4% PFA, dehydrated in 30% sucrose and embedded in Tissue-Tek O.C.T. compound (Sakura Finetek Germany GmbH). Cryosections were obtained as described previously. Sections were then blocked with PBS containing 5% bovine serum albumin (wt/vol) and permeabilized with 0.1% Triton-X 100 (vol/vol) in blocking solution. Primary antibodies were added over night at a dilution of 1:500 Iba-1 (019-19741, WAKO), 1:1,000 GFP (400-106-215, Rockland Immunocchemicals), 1:100 for MHC class II (ab23990, Abcam), 1:800 CD45 (BD Pharmingen), 1:1000 ER-TR7 (ab51824, Abcam), 1:100 CD31 (552074, BD Pharmingen), 1:500 Laminin (L9393, Sigma-Aldrich) at 4 °C. Secondary antibodies were purchased from Thermo Fisher Scientific as follows: Alexa Flour 488 1:500, Alexa Flour 555 1:500 and Alexa Flour 647 1:500 for 2 h at 20–25 °C. Nuclei were counterstained with 4',6-Diamidino-2-phenylindol (DAPI). Images were taken using a conventional fluorescence microscope (Olympus BX-61 with a color camera (Olympus DP71) or BZ-9000 (Keyence, Osaka, Japan) and the confocal pictures were taken with Fluoview FV 1000 (Olympus) using a 20× 0.95 NA (XLUMPlanFL, N, Olympus).

In general, numbers of Iba-1+ cells were quantified on a wide field microscope with a 20× magnification (Olympus BX-61 or BZ-9000). Microglia and cpMΦ were normalized to the area of the region of interest measured by using the cell-P software (Olympus) and expressed as cells mm–2. pMΦ cell counts were normalized to the vessel area measured by using the cell-P software (Olympus) and expressed either as cells mm–2. mMΦ were normalized to the length of the subdural meninges indicated by ER-TR7 immunohistochemistry and measured by cell-P software (Olympus) and finally expressed either as cells/mm. To assess MΦ labeling efficacy over time (for fate-mapping studies), YFP+ Iba-1+ cells were counted and compared to YFP+ Iba-1− cells. At least three sections of a minimum of three mice were used for each analysis.

Three-dimensional reconstruction. Free-floating 40–50-μm cryo sections from adult brain tissue were stained overnight with anti-laminin, followed by Alexa Fluor 488–conjugated secondary antibody staining, which was added at a dilution of 1:500 for 4 h at 20–25 °C. Imaging was performed on an Olympus Fluoview FV 1000 confocal laser scanning microscope using a 20x 0.95 NA objective (XLUMPlanFL, N, Olympus). Z stacks were done with 1.1-μm steps in z direction, with a xy resolution of 1,024 × 1,024 pixel. Confocal Z-Stacks were analyzed using IMARIS software 7.6 surface plug-in (Bitplane).

Electron microscopy. Animals were killed and transcardially perfused with PBS followed by 4% PFA (Serva) and 0.1% glutaraldehyde (vol/vol) (Serva) in PBS, precisely adjusted to pH 7.4. Following removal of the brain, the tissue was kept in the same fixative for 3–6 h followed by thorough rinsing in PBS and preparation of 60-μm consecutive sections using a vibrating microtome (Leica Microsystems). The sections were blocked with 3% of bovine serum albumin (Sigma-Aldrich) in Tris-buffered saline (TBS-BSA) followed by incubation of the primary antibody (goat anti GFP, 1:200, Acris antibodies) in TBS-BSA over night at 4 °C. After thorough rinsing, sections were incubated with biotinylated secondary antibodies (rabbit anti goat, 1:250, Sigma-Aldrich) in TBS-BSA for 2 h. After further rinsing, the sections were incubated with ExtrAvidin (1:100, Sigma-Aldrich) for 1 h and were finally stained with diaminobenzidine (Sigma-Aldrich) to achieve an electron dense precipitate allowing detection at the level of light as well as electron microscopy. Sections were further stained with 0.5% osmium tetroxide (vol/vol) (EMS) and 1% uranyl acetate (wt/vol) (Serva), dehydrated and embedded between coated microscope slides and cover glasses using Durcupan (Sigma-Aldrich) followed by polymerization at
After transcardial perfusion with PBS, brains were homogenized with a potter in. Whole-brain homogenate was separated by 70/37/30% layered Percoll gradient centrifugation at 800 g for 30 min at 4 °C (no brake). The CNS macroparticles containing interphase was then collected and washed once with PBS before staining. BM monocytes were isolated from tibia and femur before staining. Cells were stained with primary antibodies directed against CD11b (M1/70), CD36 (No.72-1), CD45 (30-F11), CD115 (AFS98), F4/80 (BM8) (eBioscience), CD64 (X54-5/7.1), Ly6C (AL-21) (BD Biosciences) and MHC II (M5/114.15.2) (BioLegend) at 4 °C for 20 min. Cells were washed and analyzed using a FACS Canto II (BD Biosciences) or sorted using a MoFlo Astrios (Beckman Coulter). Viable cells were gated by physical forward and side scatter pattern. Data were acquired with FACSDiva software (Becton Dickinson). Postacquisition analysis was performed using FlowJo software, version 7.6.5 (Tree Star).

Flow cytometry. After transcardial perfusion with PBS, brains were homogenized with a potter in. Whole-brain homogenate was separated by 70/37/30% layered Percoll gradient centrifugation at 800 g for 30 min at 4 °C (no brake). The CNS macroparticles containing interphase was then collected and washed once with PBS before staining. BM monocytes were isolated from tibia and femur before staining. Cells were stained with primary antibodies directed against CD11b (M1/70), CD36 (No.72-1), CD45 (30-F11), CD115 (AFS98), F4/80 (BM8) (eBioscience), CD64 (X54-5/7.1), Ly6C (AL-21) (BD Biosciences) and MHC II (M5/114.15.2) (BioLegend) at 4 °C for 20 min. Cells were washed and analyzed using a FACS Canto II (BD Biosciences) or sorted using a MoFlo Astrios (Beckman Coulter). Viable cells were gated by physical forward and side scatter pattern. Data were acquired with FACSDiva software (Becton Dickinson). Postacquisition analysis was performed using FlowJo software, version 7.6.5 (Tree Star).

Single cell RNA-seq. Microglia and perivascular macrophages were obtained from a thorough preparation of the brain cortex in which meninges and choroid plexus were removed before as described previously18. In brief, cortex (Bregma, AP: 1.54 to −1.82 mm) was dissociated into a single cell suspension. Mice were deeply anesthetized with a mixture of ketamine/xylazine (80 mg per kg; 10 mg per kg), and the brain was quickly dissected and transferred to 300-µm-thick slices. The area was dissected from each slice, and the tissue was dissociated using the papain dissociation system (Worthington) following the manufacturer’s instructions. Absence of pia was confirmed by subsequent H&E staining’s of tissue specimen. All the solutions were oxygenated for at least 10 min with a mixture of 5% CO2 in O2 (Labline). Oxygenation and a short time of dissection were crucial to keep a high rate of survival in the cell suspension. After this, the cell suspension obtained was filtered with 20-µm filter (Partec) and kept in cold HBSS solution (SIGMA) with 0.2% BSA and 0.3% glucose. Then the cells were immediately loaded in the C1 chip. Data for pvMΦ and microglia were taken from ref. 18 where they were obtained as part of single-cell analysis of more than 3,000 single cells, of which a small fraction were classified as microglia and pvMΦ. The unbiased de novo clustering was described there. Essentially, cells were clustered based on their whole transcriptome profiles, and subsequently clusters were interpreted as cell types by using cluster-specific genes of known function. Cell types were then validated in tissue sections by immunohistochemistry and single-molecule RNA FISH. pvMΦ were validated by Mrc1 and Lyve1 immuno, and microglia were identified by Aif1 (Iba-1) immuno (and being negative for Mrc1 and Lyve1).

Monocytes were isolated from BM by flow cytometry sorting for CD45, CD11b, Ly6C and CD115. Cells were then subjected to single-cell RNA-seq using the C1 AutoPrep instrument (Fluidigm) and STRT/C1 protocol, as previously described18. Each single cell was imaged and manually curated, and only single healthy-looking cells without debris were used for the analyses. Expression profiles were obtained as absolute cDNA molecule counts, and normalized to transcripts per million to compensate for differences in total transcriptome size between cell types. For t-SNE, we used the first 25 principal components, with perplexity 50 and theta = 0.5. Expressed genes can be found at http://linnarssonlab.org/cortex/

Statistical analysis. Data were tested for normality applying the Kolmogorov-Smirnov test. If normality was given, an unpaired t test was applied. If the data did not meet the criteria of normality, the Mann-Whitney U test was applied.


doi:10.1038/ni.3423