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ABSTRACT

Macrophages reside within the diverse anatomical compartments of the central nervous system (CNS). Within each compartment, these phagocytes are exposed to unique combinations of niche signals and mechanical stimuli that instruct their tissue-specific identities. Whereas most CNS macrophages are tissue-embedded, the macrophages of the cerebrospinal fluid (CSF) system are bathed in an oscillating liquid. Studies employing multi-omics technologies have recently uncovered the transcriptomic and proteomic profiles of CSF macrophages, enhancing our understanding of their cellular characteristics in both rodents and humans. Here, we review the relationships between CNS macrophage populations, with a focus on the origins, phenotypes, and functions of CSF macrophages in health and disease.

One sentence summary: We review the compartment-specific identities of the various types of tissue-embedded and fluid-immersed CNS macrophages.

INTRODUCTION

The mammalian central nervous system (CNS) consists primarily of the brain, spinal cord, and several bordering compartments (including the meninges, perivascular spaces, choroid plexus, and cerebral ventricles). Each CNS compartment hosts its own unique assortment of tissue-resident immune cells: specialised myeloid cells predominate in the healthy brain and spinal cord parenchyma, while the immune cell composition of CNS border tissues is comparatively diverse[1,2].

Macrophages are present within each CNS compartment, where they maintain homeostatic functions, respond to infection, and protect against the accumulation of cellular debris[3]. The brain parenchymal macrophages are known as microglia, while the extra-parenchymal macrophages in the CNS border structures are collectively termed CNS- or border-associated macrophages (CAMs[3] or BAMs[4], respectively - the BAM terminology is used herein). Macrophages are also present within the cerebrospinal fluid (CSF) system[5]. Initially it seemed plausible that the origins and phenotypes of CSF macrophages would resemble those of tissue-embedded BAMs. However, studies using fate mapping and multi-omics technologies suggest that some CSF macrophages may instead be more akin to microglia in terms of their ontogeny, transcriptional state, reliance on growth factors, and self-renewal capacity[1,6-8].

In this review, we describe the heterogeneity of CNS macrophages and how niche-specific signals may instruct their compartmentalised differences. We also discuss key advances regarding the origins, phenotypes, and functions of CSF macrophages.

CNS MACROPHAGE COMPARTMENTALISATION AND HETEROGENEITY

CNS macrophages are commonly separated into two location-based categories: parenchymal microglia and extra-parenchymal macrophages (**Figure 1A**). The parenchymal designation specifically refers to microglia in the functional tissue of the brain, retina and spinal cord, where neuronal and other glial cells also reside. The extra-parenchymal terminology is used for all other CNS macrophages. Compartmentalised eye macrophages have also been characterised, but are not described here (for details, see[9-10]).

Most extra-parenchymal macrophages are classed as border-associated macrophages (BAMs) because they reside within CNS border tissues, including the meningeal membranes, choroid plexus stroma, and perivascular spaces. Until recently, few markers had been identified to unambiguously distinguish parenchymal microglia from extra-parenchymal BAMs. However, single-cell RNA sequencing experiments have now revealed the compartment-specific identities of CNS macrophage subtypes in inbred C57BL/6 substrains of mice[1,11].

While microglia and BAMs share expression of pan-macrophage markers, such as *Cx3cr1*, *Csf1r*, and various complement components (e.g., *C1qb*), there are also many differences between these cells at steady state[1,11]. For instance, homeostatic microglia are enriched for *P2ry12*, *Sall1*, *Hexb*, *Siglech*, *Slc2a5*, and other genes[1,11-12]. The proteins encoded by some of these microglia signature genes function to maintain microglial homeostasis (e.g., *Sall1*[13]), while others sense changes in the parenchymal milieu (e.g., *P2ry12*[14]). Homeostatic BAMs, on the other hand, share a core transcriptional signature that is very distinct from that of parenchymal microglia. This includes signature expression of genes such as *Ms4a7*, *Ms4a6c*, *Tgfbi*, and

Lyz2[1,11] (**Figure 1B**). Notably, BAM subtypes also exhibit compartment-specific transcriptional adaptations in the healthy mouse brain.

The brain and spinal cord parenchyma are surrounded by three overlapping meningeal membranes: the dura, arachnoid, and pia mater. The thick dura abuts the skull and is considered the most permissive border, owing to its fenestrated blood vessels and vascular connections with the calvaria bones[15,16]. Intriguingly, dural BAMs can be separated into two transcriptionally distinct subsets, which can be classified as major histocompatibility class II (MHCII)^{high} and cluster of differentiation (CD)206^{low} as well as MHCII^{low}CD206^{high}(FOLR2^{high}CD38^{high}) cells, demonstrating that heterogeneous macrophage subsets can coexist *within* specific CNS border compartments[1] (**Figure 1B**). Interstitial macrophages in the lung and other peripheral tissues also consist of both MHCII^{high}CD206^{low} and MHCII^{low}CD206^{high} subsets: the MHCII^{low} macrophages preferentially localise near blood vessels, while MHCII^{high} macrophages surround nerve bundles[17]. Whether the MHCII^{high} and MHCII^{low} BAM subsets in the dura also occupy distinct microanatomical niches remains to be determined. The less permissive subdural meninges are made up of the arachnoid and pia mater, which are separated by the subarachnoid space. Compared to dural BAMs, the tissue-embedded subdural BAMs are more homogenous, consisting primarily of MHCII^{low}CD206^{hi} cells that are enriched for markers including *Lyve1, P2rx7* and *Egfl7*[1] (**Figure 1B**).

The subarachnoid space links the meninges with the CSF system, as it is filled with fluid generated mainly in the ventricles by the choroid plexus (see **Box 1**). BAMs are also present in the stroma of the choroid plexus, and these cells are enriched for genes including *Lilra5* and *Ccnd2*[1] (**Figure 1B**). As with dural BAMs, stromal choroid plexus BAMs can be subdivided into transcriptionally distinct groups of low and high MHCII-expressing cells[1,18]. In both the choroid plexus and dura, most BAMs are MHCII^{low} at

106 birth, but the fraction of MHCII^{high} BAMs increases dramatically with ageing[1,18]. IRF8
107 likely drives this phenotypic switch, as few MHCII^{high} BAMs are generated in the
108 choroid plexus of IRF8-deficient mice[1].

109 The intra-compartmental macrophage heterogeneity observed in the dura and choroid
110 plexus may be partly explained by the relative permissiveness of these compartments
111 for monocytic cells to enter the tissue and begin differentiating (due to their fenestrated
112 vasculature). Indeed, some BAMs in the dura and choroid plexus are derived from
113 monocytes in the healthy mouse brain[1,12], and monocytes and BAMs in the dura can
114 be ordered along a developmental trajectory[1]. However, despite their similar
115 ontogenies, the identities of dural and choroid plexus BAMs clearly differ[1], indicating
116 that the tissue microenvironment, or niche, also plays a role in instructing their
117 phenotypes.

118 The perivascular spaces surrounding parenchymal blood vessels are also classed as
119 border compartments, which are largely inaccessible to blood-derived cells at steady-
120 state[19]. Within these spaces, elongated macrophages known as perivascular BAMs
121 are present (**Figure 1B**). These cells also express core BAM signature genes, and are
122 enriched for markers including the interferon-inducible gene, *Bst2*[11]. The tissue-
123 specific signatures of the different BAM populations likely reflect the functional
124 specialisation of these cells, allowing them to carry out niche-specific roles.

NICHE SIGNALS BETWEEN AND WITHIN CNS COMPARTMENTS

In recent years, there has been an increasing appreciation that niche signals (such as anatomically restricted trophic factors and cell-to-cell interactions) are instrumental for attracting and maintaining macrophages and for shaping their tissue-specific identities[20]. The nature of the niche cells and signals found in the various CNS compartments are still to be fully identified, but there are some likely candidates based on observations in peripheral tissues. For instance, the identities of liver macrophages (Kupffer cells) are imprinted by anatomically-adjacent stellate cells, hepatocytes, and endothelial cells[21]. In the spleen, red pulp macrophages are maintained through the production of CSF1 by red pulp fibroblasts, which also secrete monocyte chemo-attractants upon macrophage depletion[22]. It is likely that CNS macrophage profiles are also influenced by the neighbouring cells in their micro-environments.

In the brain parenchyma, astrocytes and neurons are thought to play key roles as niche cells that drive the homeostatic profiles of microglia[23]. Microglia normally lose their ramifications and signature profiles in culture, but Baxter et al.[23] demonstrated that these phenotypes are rescued when microglia are co-cultured with both astrocytes and neurons (in part via the transforming growth factor [Tgf]-β pathway). In vivo, the parenchymal micro-environment is not uniform throughout the brain, and regional differences in microglial morphology[24], maintenance[25], profiles[26-27], and functions[28] have been described. For instance, microglia are CSF1R-dependent and the ligands for this receptor, CSF-1 and IL-34, have non-overlapping distributions in the brain[29]. Neuronally produced IL-34 predominates in forebrain regions and, accordingly, forebrain microglia primarily rely on this ligand for their development, maintenance, and transcriptomic signature[29-30]. In contrast, CSF-1 is higher in the cerebellum and cerebellar microglia are instead reliant on this ligand[29]. Intra-

compartmental differences in microglial IL-34 dependency have also been described in the retina[9].

Intriguingly, microglial enhancer landscapes and gene expression profiles also appear to be partly ontogeny-dependent[31-33]. After depletion of endogenous microglia (which are yolk sac-derived[34]), monocytes arrive in the brain parenchyma and differentiate into microglia-like cells[32-33]. However, these monocyte-derived cells lack the central *Sall1* gene module of yolk sac-derived microglia, indicating that niche signals are not the sole drivers of microglial phenotypes.

Less is known about the niche-specific cells and molecular cues that govern BAM identities, but the cellular neighbours (and therefore the niche signals) of BAMs clearly differ to those of parenchymal microglia. For instance, most cells in the choroid plexus compartment are epithelial and mesenchymal[18]. These choroidal niche cells may act to regulate BAM phenotypes in this micro-environment, as they are both sources of CSF-1[18]. Additionally, border compartments host various other immune cells, which may influence BAM phenotypes. In the choroid plexus stroma, for example, monocyte recruitment and differentiation are apparently promoted by IL-4 producing T cells[35]. Similarly, T cells in the meninges may function to prevent the pro-inflammatory skewing of meningeal myeloid cells[36]. As with the brain parenchyma, the structural niche cells within individual CNS border tissues will not be uniformly distributed, and BAM profiles may be differentially influenced depending on their precise micro-anatomical location and cellular neighbours.

It is worth considering that parenchymal microglia and extra-parenchymal BAMs are embedded within cellular networks and extracellular matrixes. This is not so for the macrophages in the CSF system, which are instead barraged by a pulsating fluid in an environment that is relatively devoid of cells. We will focus the rest of this review

175 on describing key advances regarding the macrophages of the CSF system (for
176 comprehensive reviews on microglia or tissue-embedded BAMs, see[3,37]).

BOX 1 →

THE CEREBROSPINAL FLUID SYSTEM

Anatomy and fluid turnover

Each of the four cerebral ventricles (the lateral [left and right], third, and fourth ventricles) contains an extension of the ventricular ependymal lining known as the choroid plexus, which produces CSF[38,39]. As cerebral ventricles interconnect via channels, CSF can move freely between them in a pulsatile manner that is dependent on cardiac cycle, posture, and respiration[40-41]. CSF movement is also influenced by the whip-like beating of cilia on the ventricular ependyma[42-43]. Due to these flow-inducing factors, this colourless liquid mixes within the ventricles and disperses throughout the central canal of the spinal cord and the subarachnoid cisterns and spaces.

Brain waste-removal is seemingly achieved by the CSF system. Based on the theory of a glial-lymphatic (glymphatic) system in rodents, CSF from the subarachnoid space is driven into the brain along arterial vessel walls that dip cortically from the meninges[44-45]. Upon entering the brain, it is argued that this CSF mixes with interstitial fluid and picks up extracellular waste (such as amyloid-β), which is then transferred via a paravenous return pathway back into the subarachnoid space[44-46]. From there, the collected waste can be cleared via the routes of CSF drainage.

Most classical descriptions state that CSF is reabsorbed via arachnoid granulations from the subarachnoid space into dural sinuses and ultimately back into the venous bloodstream[47]. However, recent studies have established that much of the CSF instead drains from meningeal lymphatic vessels into the peripheral lymphatic system[48-50]. Most lymphatic drainage of CSF occurs around the skull, but some also occurs at the sacral region of the spine[51]. Other exit routes for CSF, such as olfactory

pathways, have also been demonstrated[52,53]. As CSF is continuously produced, likely in a circadian manner[54], it must be drained at a similar rate to prevent its net accumulation. Based on its production rate and total volume in adult humans, this fluid is replenished 3 to 4 times per day[55,56]. In old age, the volume of CSF increases (likely because of cortical atrophy[57-58]), but its turnover rate slows, which may compromise CNS waste-removal and exacerbate neurodegenerative processes[49-50,59-60].

Cellular composition of CSF

In healthy adult humans, there are 500-3,000 cells per ml of CSF, most of which are leukocytes[61-64]. This is a low cell density compared to other sterile body fluids, making it challenging to study these cells using techniques requiring high cell input. Nevertheless, flow cytometric and single-cell analyses have shown that most CSF leukocytes are T cells, and that monocytes/macrophages, dendritic cells, natural killer cells, and other immune cells are also present[63,65-67]. The presence of diverse types of CSF immune cells may explain why the CSF system can effectively mount its own adaptive immune responses to grafts and pathogens, while the brain parenchyma can be considered an 'immune privileged' site[68]. For immune cells to exit from the CSF, meningeal lymphatic vessels can apparently traffic certain leukocytes, such as T cells and dendritic cells, from the CSF to cervical lymph nodes[48,69-70].

In healthy individuals, CSF leukocytes provide immune surveillance[56]. In cases of CNS disease, the numbers of these leukocytes can increase substantially (pleocytosis, generally considered as >5,000 leukocytes/ml). Retrospective cohort studies reveal that most cases of extreme pleocytosis (>100,000 leukocytes/ml) result from CNS infections, such as bacterial meningitis, while milder cases (5,000-50,000 leukocytes/ml) more often associate with non-infectious CNS conditions, such as demyelinating diseases[71-72].

DIFFERENCES BETWEEN CSF MACROPHAGES, BAMS, AND MICROGLIA

Locations, subtypes, and morphologies of CSF macrophages

One hundred years ago, while examining the cerebral ventricles of the frog (*Pelophylax* kl*. esculentus*), the Austrian anatomist Walter Kolmer noticed flattened, granulated cells coating the ventricle-facing surface of the choroid plexus[73]. Based on their morphologies, and as they appeared to have engulfed pigment granules from neighbouring cells, Kolmer speculated that these cells were migratory phagocytes. Following Kolmer's discovery, the existence of these cells, as well as their phagocytic nature, were confirmed in numerous vertebrate species[74-78]. Phagocytes have also been identified freely-floating within cerebrospinal fluid (CSF)[79-80] and attached to the surfaces of cerebral ventricles and subarachnoid spaces[79,81-83]. These seminal studies illustrated that phagocytes, now known to be macrophages, are present throughout the CSF system. Here, we refer to these fluid-immersed cells collectively as CSF macrophages.

CSF macrophages can be subcategorised as either freely-floating or surface-attached[5] (**Figure 1A**). Freely-floating macrophages are present throughout much of the CSF system, possibly excepting the uninjured central canal of the spinal cord[84]. The same appears true for surface-attached CSF macrophages, which can be further subcategorised based on their specific site of residence: subarachnoid space macrophages attach to the CSF-immersed surfaces of the subarachnoid blood vessels, pia mater, and arachnoid trabeculae[79,85]; supraependymal macrophages sit upon ventricular ependymal walls[82,83]; and Kolmer's macrophages sit upon the apical surfaces of the choroid plexus[5,73] (**Figure 1B**). In 1953, Kolmer's cells were renamed as 'Epiplexuszellen', or epiplexus cells, to emphasise their anatomical position,

helpfully distinguishing them from the other surface-attached CSF macrophage populations[86].

The surface features of epiplexus cells can vary greatly, with spherical, bipolar, and stellate morphologies having been reported[5]. The archetypal adult epiplexus macrophage, however, has a smooth, centrally located cell body and three to five major cytoplasmic extensions. Similar cellular morphologies have been reported for other types of surface-attached CSF macrophages, such as those on the ependymal and pial surfaces[5,76,83]. Recent *in vivo* time-lapse imaging techniques to track epiplexus cell migration have confirmed Kolmer's assumptions that these cells have a high migratory capacity, as they travel relatively large distances over choroid plexus surfaces[87].

Similarities between epiplexus macrophages and microglia

Until recently, the profiles of CSF macrophages remained largely speculative. Even within the choroid plexus, it was unknown to what extent stromal BAMs differed from epiplexus macrophages, both in terms of their ontogeny and transcriptional state. The lack of known subset-specific markers also precluded the isolation of these populations for downstream analyses. However, single-cell technologies and new genetic tools have started to provide important insights. While most choroid plexus-associated macrophages exhibit the core BAM signature in healthy mice, a smaller subset of choroid plexus-associated macrophages does not (**Figure 2**). Remarkably, these cells express microglial signature genes, including *Sall1*, *P2ry12*, and *Slc2a5* (**Figure 2B-C**). Using the *Sall1*^{CreER} fate mapping model, these macrophages were shown to be restricted to the apical surfaces of the choroid plexus epithelium, indicating that they are Kolmer's epiplexus cells[1]. McKinsey and colleagues similarly revealed labelling of a subset of macrophages on the surface of the choroid plexus

276 using *P2ry12*^{CreER} mice[88], which is another microglial fate mapper. Compared to
277 parenchymal microglia, epiplexus macrophages from healthy mice exhibited reduced
278 expression of homeostatic microglia genes (e.g., *P2ry12*, *Tmem119, and Hexb*) and
279 increased expression of genes associated with phagocytosis and lipid metabolism
280 (such as *Lpl*, *Apoe*, *Clec7a*, and *Cst7*)[1]. Intriguingly, a similar gene expression
281 signature can also be observed in parenchymal microglia that respond to inflammation
282 and disease[1,89].

Immunophenotypic profiles of human CSF macrophages

In humans, flow cytometric studies of non-diseased CSF (obtained via lumbar puncture) suggest that 5-20% of all CSF cells are monocytes[63-64,90-92]. A limitation of these studies, however, is that they typically relied on $CD14^+$ and/or $CD16^+$ to label monocytes, both of which are also expressed by certain CNS macrophages, such as reactive and plaque-associated human microglia[93-94]. Indeed, recent single-cell RNA sequencing studies of human CSF leukocytes have consistently identified a population of $CD14^+$ CSF cells that cluster separately from blood monocytes and express several BAM/microglia markers[6-7,66,95]. While numerous terms have been used to describe these cells, ranging from "microglia" to "monocytes" (**Table 1**), we refer to these cells as CSF macrophages (a term also favoured by Ramesh et al.[95]).

These $CD14^+$ CSF macrophages are present in both healthy and diseased individuals[6-7,66,95-96]. Single-cell transcriptomic profiling shows that they can express several genes expressed by homeostatic microglia, such as *CX3CR1*, *CSF1R*, *SLC2A5*, *MARCKS*, and *P2RY13*[7], as well as various genes associated with disease-associated microglia (DAMs[89]), such as *APOE*, *AXL*, and *TREM2*[6] (**Table 1**). When compared to the transcriptomes of human microglia (as described by Sankowski et al.[97]), the CSF macrophages analysed by Esaulova et al.[7] most closely resembled microglia that expressed genes associated with 'antigen processing and presentation of peptide antigen'. Based on this finding, it was suggested that CSF macrophages may act as antigen presenting cells. It has been shown that meningeal macrophages can acquire and present CSF-delivered antigens to T cells[98-99], and thus it is conceivable that CSF macrophages, which can express major histocompatibility complexes for antigen presentation[100], may similarly interact with the abundant T cell population of the CSF.

As well as expressing several microglia-associated genes, single-cell RNA sequencing studies have reported that many human CSF macrophages also express BAM-associated genes, such as *STAB1*, *CH25H*, *MRC1*, and *LYVE1*[66,96]. Of note, Heming et al.[96] identified distinct but closely related clusters of $CD14^+$ CSF cells: the cells in the largest cluster expressed BAM-associated genes (such as *MRC1* and *LYVE1*), while the cells in the smallest cluster expressed microglia-associated genes (such as *P2RY12* and *TMEM119*) (**Table 1**). Transcriptomic comparisons indicated that the global profiles of both clusters partially resembled human microglia. Notably, the transcriptomes of the BAM marker-expressing CSF macrophages also resembled blood monocytes[96], which agrees with a single-cell mass cytometry study that identified some similarities between $CD206^+$ CSF macrophages and peripheral blood mononuclear cells[27].

A possible limitation of these studies is that blood contamination is common during lumbar puncture. As the concentration of blood cells is far greater than that of CSF, any contamination of CSF by blood could potentially result in skewed, misleading data. Most single-cell studies of human CSF do not report whether samples were excluded based on blood contamination, apart from two reports that rejected samples with >200 red blood cells/μl[66,96] (**Table 1**). Another limitation is that the CSF analysed in these single-cell studies was predominantly derived from diseased individuals. Even the individuals classified as controls often had morbidities (for example, subclinical neuroinflammation, hepatitis C infection, and idiopathic intracranial hypertension), so these studies may more accurately depict the nature of CSF myeloid cells in disease rather than in good health.

Collectively, these transcriptomic studies have shown that CSF macrophages express many genes associated with microglia, BAMs, and monocytes. Yet, while similarities

exist between CSF macrophages and other myeloid cell types, their global profiles still clearly differ from all other cell types. Based on their transcriptomic and proteomic signatures, as well as their extra-parenchymal localisation, we suggest that CSF macrophages should be classified as a separate population of brain macrophages, in addition to parenchymal microglia and tissue-embedded BAMs. Lineage tracing will be necessary to further define the relationship between CSF macrophages and the other brain myeloid cell populations.

Table 1. Single-cell profiling studies of human CSF macrophages. Like many macrophage populations, CSF macrophages abundantly express complement (*C1Q*) and human leukocyte antigen (*HLA*) genes. These macrophages also express certain genes associated with homeostatic/reactive microglia, border-associated macrophages, and monocytes. The information provided in this table refers generally to the cell cluster(s) as described in the respective references, and does not refer to cells derived specifically from control or diseased individuals. MS, multiple sclerosis; HIV, human immunodeficiency virus.

Reference	Donor cohort	Single-cell profiling technology used	Criteria for blood contaminaton-based sample exclusion	Term used for cells in cluster	Example highly expressed genes	Comments on transcriptomic signature	PMID
Beltrán et al., 2019[173]	4 MS-discordant monozygotic twin pairs, 4 clinically healthy co-twins with signs of subclinical neuroinflammation	scRNAseq (Smart-Seq2)	not reported	monocytes	*C1QA/B/C, CD14, CSF1R, MS4A7, CD68, TGFβ1, TREM2, CD163*	no specific comments	31566584
Farhadian et al., 2018[6]	3 HIV+ donors, 2 HIV-uninfected donors	scRNAseq (SeqWell)	not reported	myeloid-2 / microglia-like cells	*C1QA/B/C, TREM2*, *APOE*, *AXL*	"We found significant overlap between genes that are overexpressed in Myeloid-2 and genes that are enriched in neurodegenerative disease–associated microglia"	30232286
Esaulova et al., 2020[7]	2 donors with MS, 1 with anti-MOG disorder, reanalysed 2 HIV+ patients from Farhadian et al., 2018	scRNAseq (10X Genomics)	not reported	CSF microglia	*CX3CR1, CSF1R, SLC2A5, MARCKS, and P2RY13*	"cells with a transcriptomic signature matching microglia", "high expression of genes from the microglial homeostatic gene signature"	32371549
Ramesh et al., 2020[95]	12 donors with MS, 1 other neurological diseases, 3 healthy controls	scRNAseq (10X Genomics)	not reported	CSF macrophages	*APOE, FCER1G, IFITM3, C1QA/B/C, FTH1, and various HLA genes*	"The myeloid population was also distinct [in CSF compared to blood], with blood CD14+ and CD16+ monocytes existing in a transcriptional continuum with what we broadly termed CSF macrophages"	32859762
Schafflick et al., 2020[66]	6 donors with idiopathic intracranial hypertension as controls, 6 MS	scRNAseq (10X Genomics)	red blood cell count >200/µl in CSF	mono2 / monocytes	*CD14, TREM2, TMEM119, GPR34, STAB1, CH25H, and LYVE1*	"resembled homeostatic microglia"	31937773
Heming et al., 2021[96]	8 donors with COVID-19 infection with neurological manifestations, 5 viral encephalitis, 9 MS, 9 idiopathic intracranial hypertension	scRNAseq (10X Genomics)	red blood cell count >200/µl in CSF	mono1	*CD14, MRC1*, *LYVE1*	"the mono1 cluster preferentially expressed CNS-border associated macrophage genes"	33382973
				mono3	*CD14, SPP1, OLFML3, P2RY12, TMEM119*	"the mono3 cluster expressed known microglia-associated genes"	
Böttcher et al., 2019[27]	4 male donors, 39-61 years of age	single-cell mass cytometry (CyTOF)	not reported	CSF mononuclear cells	CD14, CD206, CD163, IL6, CCR2, CCR7, CD68	no specific comments	30559476

DEVELOPMENTAL ARRIVAL OF BRAIN AND CSF MACROPHAGES

Most of our knowledge about CSF macrophage arrival derives from murine studies, so the following section focuses on mouse development (unless otherwise stated). In mice, the earliest version of the CSF system forms by embryonic day (E)9-9.5 as the neural tube zips shut, trapping amniotic fluid within the tube[101]. After shutting, the cephalic end of the neural tube dilates and forms the three primary brain vesicles: the prosencephalon (forebrain), mesencephalon (midbrain), and rhombencephalon (hindbrain). As development continues, these expanding primary vesicles become the five secondary vesicles (the prosencephalon subdivides into the telencephalon and diencephalon, while the rhombencephalon subdivides into the myelencephalon and metencephalon). Following neural tube closure, choroid plexus tissue emerges in each of the developing ventricles. This tissue arises first in the fourth ventricle (at approximately E11.5), then concurrently in both lateral ventricles (at approximately E12), and finally in the third ventricle (at approximately E13.5, eventually fusing with the lateral ventricle choroid plexus). The subarachnoid space forms when a meshwork of loosely organised primitive meningeal cells undergo gradual cavitation, with its boundaries being defined during a period of delineation from E14-16[102].

The earliest macrophages begin arriving in the embryo proper from E9.5[34,103-104], following the stepwise establishment of the embryonic circulation[105]. In the head of the embryo, these early macrophages first colonise the cephalic mesenchyme, and then start to invade the developing CNS parenchyma[8,34,106-107]. The first macrophages within the developing CSF system, however, are not detected until E10.5[8,80] (**Figure 3A**). Intracerebroventricular IBA1^{+} cells likely arrive at equivalent developmental stages in humans[108] (Carnegie stage 13). Most or all the early CSF macrophages in

mouse express the haematopoietic markers c-KIT, Runx1, and Ly6a[109]. Of note, many of these cells also express CD206[8] (**Figure 3B**).

There is limited information regarding the arrival mode of CSF macrophages, but *ex vivo* time-lapse imaging data show that macrophages can enter ventricular lumens by squeezing across ventricular walls in embryonic slice cultures[8]. Many of the earliest CSF macrophages localise around the thin and membranous roof of the fourth ventricle in zebrafish and mouse[8,110], which may represent an early entrance site for these cells. Whatever their mode of entry, CSF macrophages, which are present individually or in cell clusters, continually increase in number following their initial arrival in the developing embryo[8,77,80].

The earliest arrival of CSF macrophages is reliant on a conserved super-enhancer within the colony stimulating factor 1 receptor (*Csf1r*) locus[8], known as the fms intronic regulatory element (FIRE). Deletion of FIRE also results in the failure of primitive microglia to seed the brain parenchyma during development, whereas it causes only a partial decline in the arrival of BAMs[8,111].

It has been speculated that CSF macrophages spread throughout the CSF system by detaching from, and reattaching to, tissue surfaces[75]. Although this concept requires validation, experimental evidence supports the idea that macrophages and other cells can migrate throughout the CSF system and fasten themselves to tissue surfaces[69,112].

Most CSF macrophages are rounded cells early in their development, but they progressively ramify as embryogenesis proceeds[77]. Their increasing ramification likely signifies cellular maturation[5,77,113] and may facilitate their secure anchorage to their respective tissue surfaces[114].

ORIGINS OF BRAIN AND CSF MACROPHAGES

Sources of macrophages

Macrophages are generated from multiple progenitor sources, which have been studied extensively during mouse embryogenesis. The earliest of these progenitors are found in the blood islands of the mouse yolk sac from E7.25[115-116]. These are primitive haematopoietic progenitors that can produce immature/nucleated erythroid, megakaryocytic, or macrophage lineage cells[115,117-119]. Based on current knowledge, macrophages and primitive erythroid cells are generated in parallel during this initial haematopoietic wave from apparently distinct, lineage-restricted progenitors[115-117,119]. As there is currently no evidence that individual primitive progenitor cells have *both* erythroid and myeloid lineage potential, some argue that they should not be considered as erythro-myeloid progenitors (EMPs).

The yolk sac also provides the second source of macrophage progenitors. These yolk sac progenitors emerge from hemogenic endothelium via endothelial-to-hematopoietic transitions in blood islands and vessels[120]. The second wave progenitors are multipotent EMPs, as they can differentiate into definitive/enucleated erythrocytes, megakaryocytes, macrophages and other myeloid lineages, but lack the potential of haematopoietic stem cells (HSCs) to form lymphoid progenitors, such as B cells[119]. After their generation in the yolk sac from E8.5, many EMPs (of which, there may be heterogenous populations) emigrate via the vasculature to the fetal liver, where they rapidly expand and differentiate[121-122].

A third source of macrophage progenitors are HSCs, which are capable of repopulating both myeloid and lymphoid lineages in adult mice. HSCs are initially generated from intra-aortic hematopoietic cluster cells in the aorta-gonad-mesonephros region of the embryo at E10.5[123-125]. Intra-aortic hematopoietic clusters

emerge from hemogenic endothelium in the major arteries, again via endothelial-to-haematopoietic transitions[125-127]. By E11, HSCs are also present in the yolk sac, fetal liver, placenta, and possibly the head[123-125,128-130]. HSCs (and at least some EMPs) transition through monocytic intermediate phases as they differentiate into macrophages. Near birth, HSCs seed the bone marrow, which becomes the major site of haematopoiesis in adults. These processes of murine macrophage development appear to be somewhat conserved in humans[131-132], but our knowledge of human macrophage origins is limited.

Ontogeny and turnover of brain and CSF macrophages

The ontogenies of certain CNS macrophage populations have been explored in detail in mouse (see[1,12,34,106-107,133]). These studies show that microglia are yolk sac-derived cells that are maintained through self-replication in normal health. However, the precise contributions of the different progenitor waves to the adult pool of microglia is debated[116,134]. Indeed, there are data suggesting that each of the three embryonic progenitor waves (primitive progenitors, EMPs, and HSCs) may contribute[34,121,133]. BAMs also initially derive from yolk sac-generated progenitors during embryogenesis[107], but in the dura and choroid plexus many of these cells are ultimately replaced by monocyte-derived macrophages in adulthood[1,12]. Recent findings suggest that some of the monocyte-derived macrophages in the dura arise from myeloid cell reservoirs in the adjacent skull bone marrow, rather than from circulatory routes[16]. It is unclear whether skull and vertebral bone marrow also supply other BAM subpopulations, such as stromal choroid plexus macrophages. CSF macrophage ontogenies were largely ignored until recent elaborate studies provided details concerning the origins and turnover of epiplexus macrophages on the choroid plexus surface[1,12].

Despite choroid plexus epithelia being linked by specialised junctions from early in their development[135], some researchers have claimed that macrophages can enter ventricles by migrating between choroidal epithelia (via paracellular migration [136]). In contrast, others have argued that this cell migration occurs via a rare process known as emperipolesis, whereby a macrophage would enter the cytoplasm of a choroidal epithelial cell from its stromal surface, before migrating through the cell and exiting its ventricular surface[76,137-138]. In the adult monkey, both electron and scanning electron microscopy images were published to sustain this claim of emperipolesis[137-138]. However, evidence contradicting this view was recently published by Van Hove et al.[1], who used multiple techniques to explore the lineages and turnover of various CNS macrophage populations.

Using pulse-chase labelling with the *Cx3cr1*^{CreER}:R26-YFP mouse model, time-dependent loss of YFP labelling in CNS macrophages was examined (loss of labelling over time indicates turnover by peripheral cells, such as circulating monocytes, while label retention suggests self-maintenance/longevity of a given macrophage population). Whereas microglia labelling remained stable, indicating that these cells do not receive significant input from peripheral cells, labelling of stromal choroid plexus BAMs gradually declined over time, suggesting that these cells are indeed replaced from peripheral cells. The results for epiplexus macrophages demonstrated that, like microglia, these cells retained YFP labelling over time, indicating that they self-replicate and/or are long-lived, rather than being replaced by peripheral/circulating cells over time[1]. In addition, Van Hove et al.[1] used the *Flt3*^{Cre}:R26-YFP model to examine which populations of brain macrophages are turned over by Flt3-dependent hematopoietic precursors. This model allows the distinction between Flt3-dependent bone marrow-derived circulating monocytes (YFP^{+}) and Flt3-independent embryonic

progenitors (YFP^-). As anticipated, few microglia were YFP-labelled at any time point, suggesting that they are not turned over from bone marrow-derived circulating monocytes. YFP^+ labelling of stromal choroid plexus BAMs increased over time, again suggesting their gradual turnover by monocytes with age. As with microglia, few epiplexus macrophages were YFP-labelled at any time-point during the study[1]. Finally, Van Hove et al.[1] performed macrophage depletion/repopulation experiments using the CSF1R inhibitor, PLX3397, along with fate-mapping experiments to lineage trace the repopulating cells (with both *Cx3cr1*^{CreER}:R26-YFP and *Sall1*^{CreER}:R26-YFP mice). The macrophage depletion experiments almost entirely depleted all CNS macrophage populations. After their repopulation, microglia were YFP labelled in both models, indicating that they repopulate fully via local self-renewal. In contrast, the repopulated stromal choroid plexus BAMs exhibited a significant reduction in YFP labelling, suggesting that they at least partly relied on peripheral input for their repopulation. Again, like microglia, the repopulated epiplexus macrophages were YFP-labelled in both models. Epiplexus macrophages self-renewed from pre-existing *Sall1*$^+$ progenitors, rather than from the *Sall1*$^-$ cells that repopulated the BAMs of the choroid plexus stroma[1] (**Figure 3C**). Consistent with these data, a recent *in vivo* time-lapse imaging study reported that macrophages were not observed crossing from the choroid plexus to the CSF under baseline conditions or in response to experimental inflammation in mice[87].

CSF MACROPHAGES IN HEALTH AND DISEASE

Based on their transcriptomes, CSF macrophages have been linked with many biological processes, such as lipid metabolism, stimulus detection, antigen presentation and phagocytosis[1,7]. In support of these links, studies show that CSF macrophages are highly capable at recognising and engulfing various types of molecules/cells both *in vitro*[1] and *in vivo*[75,77-79]. Their phagocytic capacity was explored experimentally by Carpenter et al.[75], who demonstrated that tracers (Indian ink, thorotrast, and ferritin) injected directly into the cerebral ventricles of cats were phagocytosed within minutes by epiplexus macrophages. Subsequent reports demonstrated that CSF macrophages can also engulf blood serum-derived substances[77-78]. In these studies, rhodamine isothiocyanate and horseradish peroxidase were injected into the vasculature of rats and these tracers were subsequently shown to be taken up by CSF macrophages, usually within hours of injection. These blood-injected tracers were seemingly distributed into the CSF from the choroid plexus[5,78]. Conversely, intra-peritoneal injection of a larger tracer, Texas red-conjugated dextran (70 kDa), did not result in its uptake by murine epiplexus macrophages[87], consistent with the blood-CSF barrier being somewhat size selective[139]. In agreement with these cells being expert phagocytes, epiplexus macrophages far outperform microglia in their ability to internalise pHrodo-labelled *Escherichia coli* bioparticles *in vitro*[1].

Regardless of their roles in normal health, mutant mice lacking CSF macrophages (while selectively retaining some other tissue-resident macrophage populations) can survive well into adulthood[8,111]. This finding, which indicates that CSF macrophages are not absolutely required for normal development or survival, is perhaps unsurprising. Assuming that their major roles are to survey the fluid and clear

516 exogenous materials from the CSF system, their functional importance would likely
517 become increasingly apparent when CSF homeostasis is interrupted.

518 **Hydrocephalus and ventricular enlargement**

519 In cases where CSF homeostasis fails, fluid can accumulate in the cerebroventricles
520 and subarachnoid spaces causing hydrocephalus. In resource-poor countries,
521 hydrocephalus primarily occurs due to infection; in resource-rich countries, it mostly
522 occurs due to haemorrhage[140-141]. In both post-infectious and post-haemorrhagic
523 hydrocephalus, inflammation is thought to drive the pathological features.

524 Studies consistently show that CSF macrophages become more numerous and are
525 activated in cases of hydrocephalus[142-147]. In rodents, this CSF macrophage response
526 has been described in prenatal rats with 6-aminonicotinamide-induced
527 hydrocephalus[142-143] and in adult spontaneously hypertensive rats, which develop
528 post-haemorrhagic hydrocephalus[144-145]. Increased CSF macrophage numbers and
529 activation has also been described in fetal and childhood cases of hydrocephalus in
530 humans[146-147]. In contrast, the transcriptomic landscape of human microglia in
531 hydrocephalus was comparable to controls in a recent single-cell RNA sequencing
532 study[148]. In cases of fetal hydrocephalus, CD68-expressing CSF macrophages
533 accumulated along the ependymal walls of ventricles, while these cells were absent in
534 non-hydrocephalic control fetuses[147]. Accumulation of lipid-droplet-containing CSF
535 macrophages has also been described in childhood hydrocephalus, with higher
536 proportions of these fat-laden phagocytes being associated with worse outcomes[146].
537 In post-haemorrhagic hydrocephalus, factors secreted into the CSF can actively
538 promote hydrocephalus development. Indeed, injection of acellular CSF from post-
539 haemorrhagic hydrocephalic human patients into the ventricles of adult nude mice
540 resulted in ventricular enlargement as well as epiplexus macrophage activation (when

compared to mice injected with acellular CSF from a control patient[149]). Thrombin is an example of a molecule with increased activity in the CSF following haemorrhage, which can directly stimulate hydrocephalus[150]. Injection of thrombin into the ventricles of non-hydrocephalic rats caused increased numbers and activation of epiplexus macrophages (in comparison to saline-injected controls), indicating that it can also stimulate CSF macrophages in hydrocephalus[145]. Increased intracranial pressure alone can similarly enhance the numbers and activation of epiplexus macrophages, as shown via the injection of artificial cerebrospinal fluid into the cisterna magna of rats[151]. Thus, intracranial pressure and blood degradation products, alone or in combination, can stimulate CSF macrophages in hydrocephalus.

Intriguingly, hydrocephalus is one of the most consistent phenotypes observed in animals lacking macrophages due to mutations in *Csf1r*. For example, postnatal *Csf1r*$^{-/-}$ mice on the C57BL/6N background display progressive ventricular enlargement, particularly in the lateral ventricles, with corresponding compression of the surrounding brain parenchyma[152]. Furthermore, ventricular enlargement has been described in one year old haploinsufficient *Csf1r*$^{+/-}$ mice[153], in *Csf1r*-deficient rats[154], and in humans with bi-allelic *CSF1R* mutations[155]. It has been speculated that cellular debris may accumulate in CSF in the absence of macrophages, causing interference of normal outflow and consequent ventricular expansion[152]. However, the causes and features of hydrocephalus when macrophages are absent will differ when compared to post-infectious/post-haemorrhagic forms of hydrocephalus. Future studies are undoubtably required to better understand the mechanistic links between macrophage loss and hydrocephalus.

Subarachnoid haemorrhage

In subarachnoid haemorrhage, erythrocytes stream into the CSF of the subarachnoid space, which is associated with brain injury and cerebral ischemia[156]. Some of the extravasated erythrocytes are cleared from the CSF via meningeal lymphatic vessels[157], while others are promptly phagocytosed and subsequently digested by CSF macrophages. Consequently, CSF macrophage cytology can be used for diagnosis of subarachnoid haemorrhage in difficult to detect cases that are CT scan-negative[158]. CSF macrophages that have engulfed erythrocytes, but have not yet digested them, are known as erythrophages (these cells indicate a recent haemorrhage[159]). CSF macrophages that have engulfed *and digested* erythrocytes will stain positive for hemosiderin, an iron-storage complex, and are known as siderophages (these cells indicate an older haemorrhage[160]). This example illustrates how CSF macrophages can provide information about both the type and timing of pathology.

In both rodents and humans, CSF macrophages increase in numbers and become activated in subarachnoid haemorrhage[145-147,149-151]. A recent flow cytometry study demonstrated that expression of CD163 is increased in CSF macrophages in human subarachnoid haemorrhage patients compared to patients with unruptured aneurysms[161]. CD163 functions as a haemoglobin scavenger receptor involved in blood clearance after subarachnoid haemorrhage[162-163]. It was further shown that CD163 expression by CSF macrophages correlated with higher CSF bilirubin concentrations and worse grades of subarachnoid haemorrhage (after controlling for age and sex[161]). Notably, increased CD163 expression was associated with improved outcomes in severity-matched individuals[161].

Infection

589 Although barrier systems protect the CNS from infection, certain pathogens can
590 breach these defences. Once established, CNS infections commonly cause CSF
591 pleocytosis, with different pathogens having distinct effects on the composition of the
592 CSF leukocyte population[164]. During a typical CNS infection, CSF pleocytosis will peak
593 shortly after infection, followed by a gradual normalisation of cell numbers as
594 inflammation settles. Some CSF leukocytes undergo apoptosis during the resolution
595 of inflammation and are cleared by CSF macrophages[165]. Cytodiagnostic studies have
596 demonstrated that CSF macrophages can uptake various types of pathogens (such
597 as fungi, bacteria, viruses, and parasites), and many terms have been coined to
598 categorise these cells based on their substrates of phagocytosis.
599 An example of a pathogen that can invade the CNS is *Mycobacterium tuberculosis*.
600 Entry of this bacterium into the CNS can cause tuberculous meningitis, which is more
601 common and less treatable in immunocompromised individuals[166-167]. In nearly all
602 cases of tuberculous meningitis, the bacteria enter the subarachnoid space[168], where
603 they are found abundantly within humans CSF macrophages[169]. The responses of
604 CSF macrophages to an attenuated non-tuberculous strain of mycobacterium, known
605 as bacillus Calmette-Guérin, were demonstrated in a series of publications in which
606 these microbes were injected into the cisterna magna of dogs[85,170-171]. These studies
607 used electron microscopy to highlight the vast expansion of CSF macrophages on the
608 surfaces of the subarachnoid space, ventricles, and choroid plexus, finding an
609 approximately tenfold increase in epiplexus macrophages three days after injection[170].
610 The consequences of maternal immune activation on embryonic epiplexus
611 macrophages have recently been explored in mice via injection of a viral genome
612 mimetic, polyinosinic-polycytidylic acid[172]. Administration of this immunostimulant to
613 pregnant dams at E12.5 caused accumulation of rounded IBA1^{+}CD68^{+} epiplexus

614 macrophages on the choroid plexus surfaces of the embryos by E14.5[172]. Simulation
615 of maternal immune activation in this manner also increased the concentration of C-C
616 motif chemokine ligand 2 (CCL2) in the embryonic CSF. Consequently, the
617 researchers injected CCL2 into the embryonic cerebral ventricles (*in utero*) to explore
618 whether this chemokine impacts choroid plexus immune cells, such as epiplexus
619 macrophages. Injection of mouse recombinant CCL2 alone was enough to cause an
620 accumulation of epiplexus macrophages on the embryonic choroid plexus surface
621 within a day of its administration[172]. Using two-photon time-lapse imaging, the authors
622 went on to show that intracerebroventricular administration of a CCL2-encoding
623 adeno-associated virus caused enhanced motility of epiplexus macrophage cellular
624 projections. Furthermore, immunohistochemistry data indicated that increased CSF-
625 CCL2 stimulated epiplexus macrophage proliferation and it was claimed that this also
626 resulted in macrophages crossing from choroid plexus to CSF[172]. However, their claim
627 of macrophages breaching across the choroid plexus was based on the locations of
628 these cells in static images.
629 These studies illustrate that CSF macrophages are not passive bystanders in CNS
630 infections. However, it remains unclear under which conditions their functions are
631 beneficial or detrimental to disease outcomes.

CONCLUSIONS

Recent methodological and technical advances have facilitated the rapid characterisation of the transcriptional diversity of CNS macrophages. However, we still know little about the niche-specific signals that instruct their compartmentalised differences in the healthy CNS. We also do not know to what extent CNS macrophages reciprocally instruct the profiles of their niche neighbours in the brain and its borders compartments (for additional open questions regarding BAMs and microglia, see[3,37]).

A century on since the earliest sketches of CSF macrophages were produced, our knowledge of these cells has improved substantially, but considerable gaps are also still present. For example, we do not know whether CSF macrophages play roles in maintaining blood-CSF barrier function or whether they may regulate the contents of the fluid under homeostatic conditions. To begin addressing these questions, future studies could contrast the proteomic and metabolomic signatures of CSF in the presence versus the absence of these phagocytes. Additionally, there are many unknowns regarding the ontogeny and spatial relationships between CSF macrophages residing in different anatomical sites, such as: What are the progenitors of supraependymal, epiplexus, subarachnoid space, and freely-floating CSF macrophages? Do CSF macrophages self-renew *in situ*, or do they require input from adjacent sites? Can epiplexus macrophages detach from the choroid plexus, float away in the fluid, and become subarachnoid macrophages? Can CSF macrophages transverse ependymal walls throughout life to interact/exchange with the tissue-embedded macrophages of the CNS (and vice versa)? Are CSF macrophages drained via the lymphatic routes of CSF disposal? How do CSF macrophages interact with other CSF leukocytes? Understanding the spatial dynamics of CSF macrophages may clarify some unknowns regarding their cellular origins and their recruitment in disease.

As we learn more about the functional significance of CSF macrophages in the years to come, it may become possible to develop therapeutic strategies to target these clinically-accessible, fluid-immersed macrophages.

Figure legends

Figure 1. CNS macrophage locations, terminologies, and compartment-specific identities. (**A**) The types and locations of parenchymal and extra-parenchymal CNS macrophages. (**B**) Macrophage compartmentalisation in the CNS. Example key markers are shown to demonstrate the compartment-specific identifies of homeostatic CNS macrophages. Freely-floating and surface-attached macrophages are present in both the subarachnoid space and the cerebral ventricles. Note that only the vasculature of the dura and choroid plexus are fenestrated, which is proposed to make these compartments relatively permissive to immune cell entry. Blood-derived and self-renewal arrows are based on our current understanding of CNS macrophage turnover. ChP, choroid plexus. MΦ, macrophage; BAM, border-associated macrophages; TE, tissue-embedded.

Figure 2. Single-cell RNA sequencing reveals compartment-specific macrophage identities of choroid plexus-associated macrophages. (**A**) Single-cell RNA sequencing was performed on immune cells isolated from the healthy adult mouse choroid plexus. From this dataset[1], monocytes and macrophages were selected and re-analysed. The UMAP projection shows the main macrophage subsets that are observed in the homeostatic ChP, with the majority being stromal macrophages (red), and a smaller subset of epiplexus macrophages (green). This Figure was generated through re-analysis of data from[1]; code and information to replicate this figure are available at GitHub* (**will be made available prior to publication*). (**B**) Volcano plot displaying differentially expressed genes (red) by epiplexus macrophages compared to stromal choroid plexus macrophages. (**C**) Feature plots showing key differentially expressed genes between epiplexus and stromal choroid plexus macrophages. Stromal choroid plexus macrophages highly express core BAM signature genes, while epiplexus macrophages highly express many microglia signature genes. MΦ, macrophage.

Figure 3. Development and origins of CSF and choroid plexus macrophages in the mouse. (**A**) Developmental milestones relating to CNS and CSF macrophage arrival. (**B**) Arrowheads show examples of CD206 and IBA1 co-stained cells in the fourth ventricle lumen of an E11.5 mouse embryo. Many early CSF macrophages express CD206, IBA1, c-KIT, Runx1, and Ly6a[9,122,123]. Scale bar, 50 µm. (**C**) Cross sectional representation of the choroid plexus, which has modified ependymal cells that form a blood-cerebrospinal fluid barrier. Embryo-generated stromal ChP macrophages (yellow cells) are gradually replaced over time by bone marrow-derived cells, which likely enter the stroma via diapedesis through the fenestrated choroidal vasculature. Conversely, epiplexus macrophages (blue cells) are maintained via the self-renewal of embryo-generated macrophages.

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A **Types and locations of CNS macrophages**

Extra-parenchymal CNS MΦ

CSF MΦ

Surface-attached

Perivascular MΦ
(in perivascular space
surrounding blood vessels)

Stromal ChP MΦ

Meningeal MΦ

Dural MΦ

Subdural MΦ

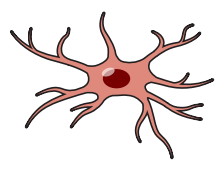
Subarachnoid space MΦ

Kolmer's epiplexus MΦ
(on CSF-exposed
surfaces of ChP)

Supraependymal MΦ
(on ependymal walls)

Freely-floating CSF MΦ

Microglia
(in brain/
spinal cord
parenchyma)



B **Macrophage compartmentalisation in the CNS**

Meninges

Parenchyma

Dural MΦ
Ms4a7^{+} Tgfbi^{+}
Lyz2^{hi}

Monocyte

MHCII^{hi}
Cd74^{hi}

Mrc1^{hi}
Cd163^{+} Clec4n^{hi}
Folr2^{hi} Ccl8^{+}

Dura

Arachnoid

Subarachnoid
space MΦ

Subarachnoid space
(CSF-filled)

Pia

TE subdural MΦ
Ms4a7^{+} Tgfbi^{+} Lyz2^{hi}
Lyve1^{hi} Mrc1^{hi}
Cd163^{+} P2rx7^{hi}

Perivascular MΦ
Ms4a7^{+} Tgfbi^{+} Lyz2^{hi}
Lyve1^{hi} Mrc1^{hi}
Cd163^{+} P2rx7^{hi}

Brain parenchyma

Ependyma

Epiplexus MΦ
Sall1^{int} P2ry12^{int}
Tmem119^{int} Cst7^{+}
Clec7a^{+} Gm1673^{+}

Choroid
plexus

MHCII^{hi}
Cd74^{hi}

Cerebral ventricle
(CSF-filled)

Microglia
Sall1^{hi} P2ry12^{hi}
Tmem119^{hi}

Supraependymal MΦ

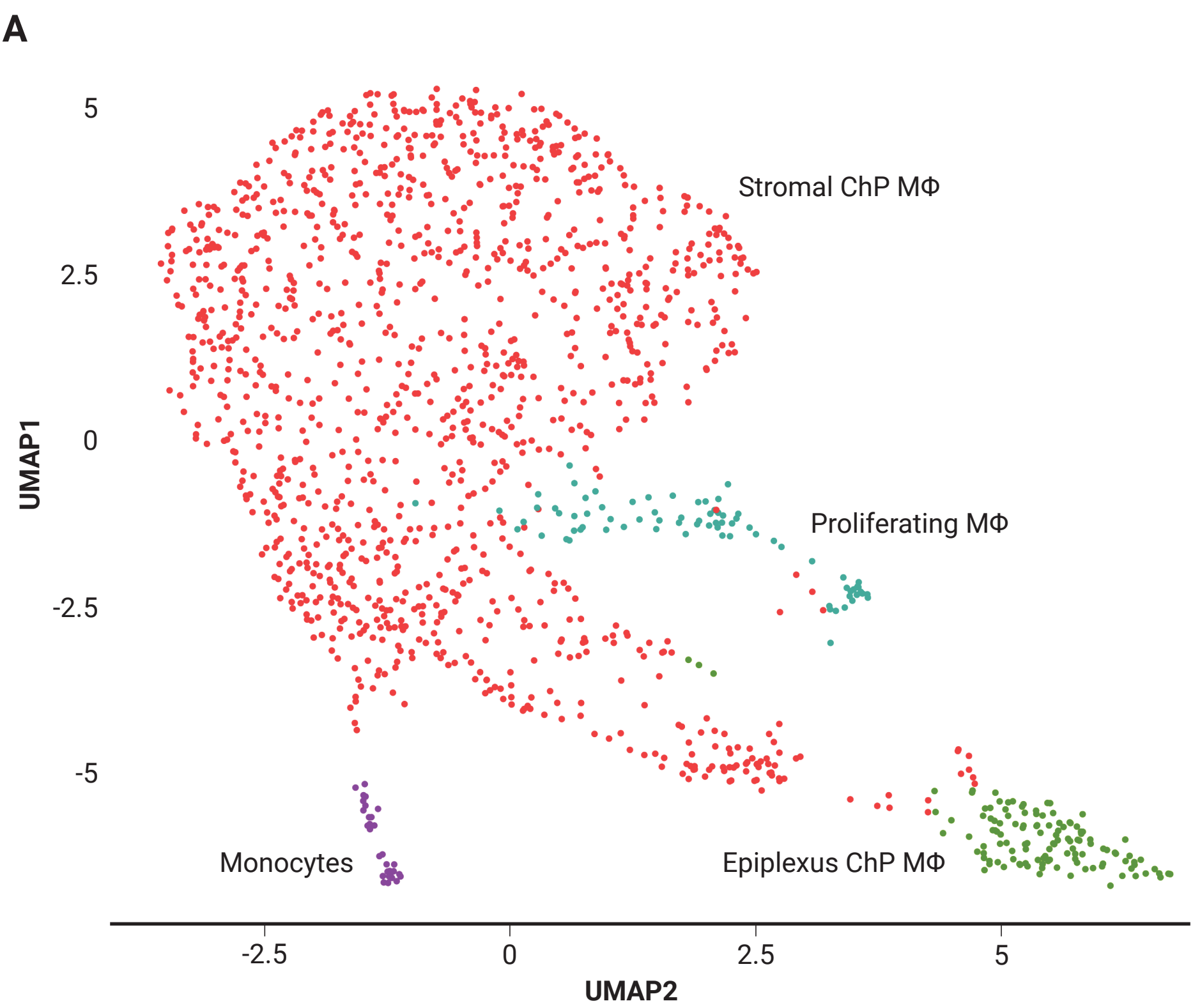
Mrc1^{int}
Cd163^{+}
Clec4n^{int}

Stromal ChP MΦ
Ms4a7^{+} Tgfbi^{+}
Lyz2^{hi} Lilra5^{+}

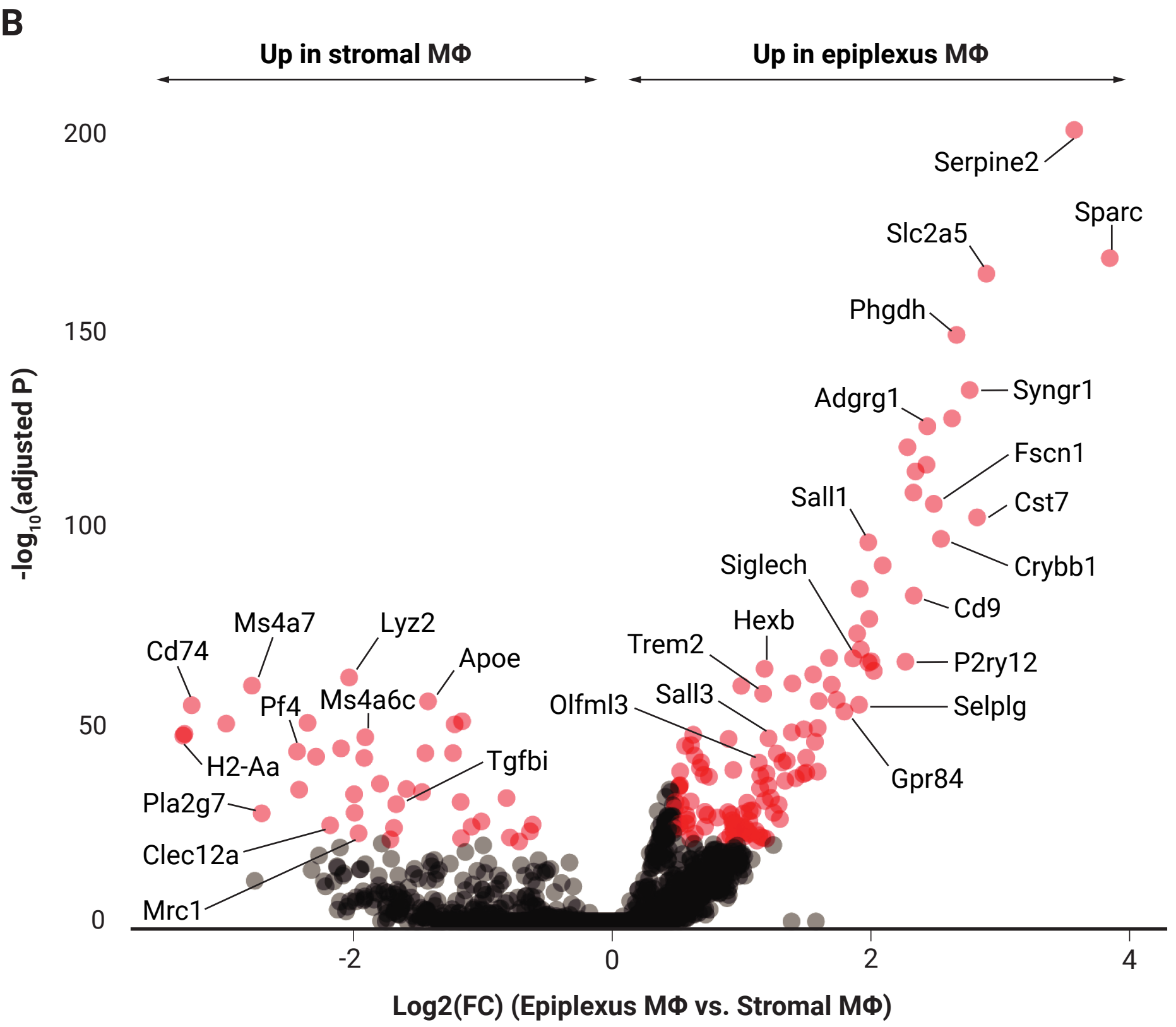
Freely-floating CSF MΦ

ChP, choroid plexus; MΦ, macrophage; TE, tissue-embedded

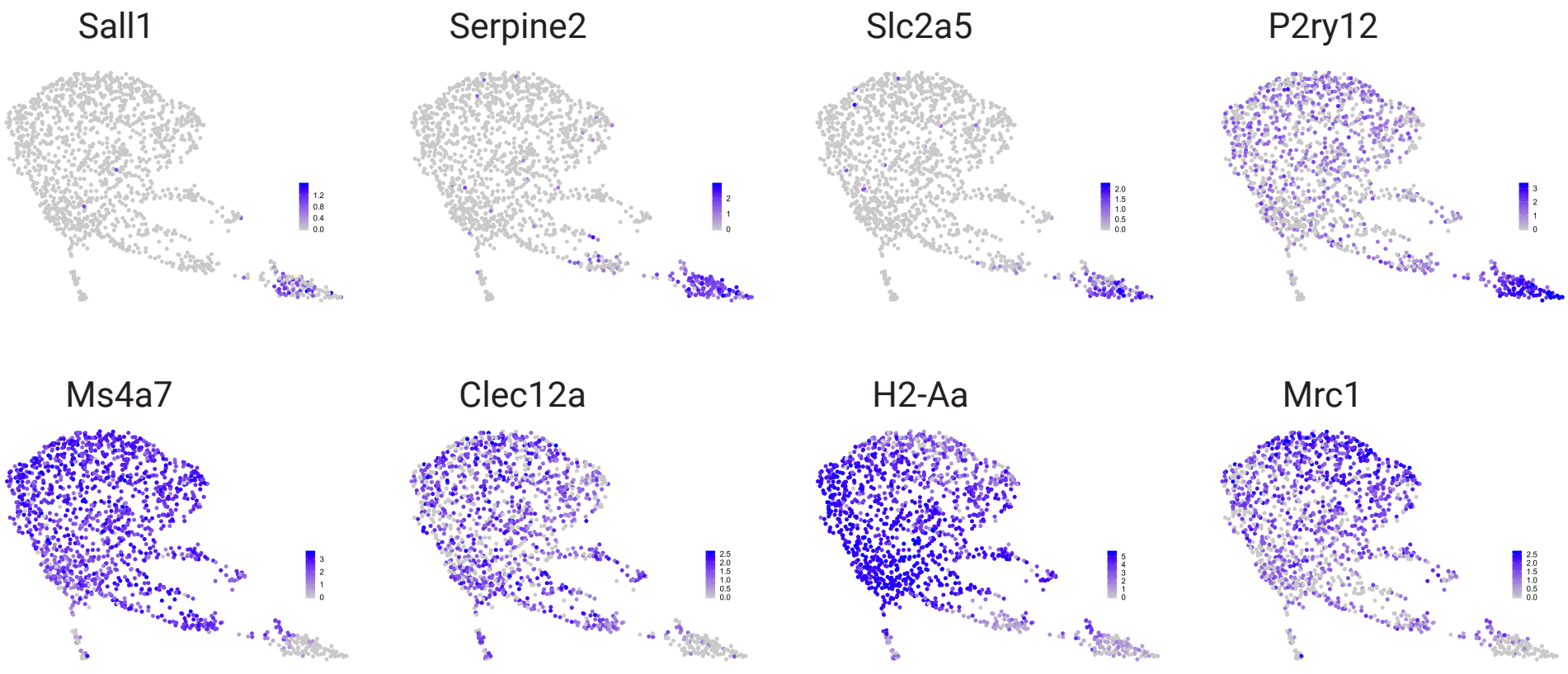
A


5
2.5
0
-2.5
-5
UMAP1
Stromal ChP MΦ
Proliferating MΦ
Monocytes
Epiplexus ChP MΦ
-2.5
0
2.5
5
UMAP2

B


Up in stromal MΦ
Up in epiplexus MΦ
200
150
100
50
0
-log10(adjusted P)
Serpine2
Sparc
Slc2a5
Phgdh
Adgrg1
Syngr1
Fscn1
Sall1
Cst7
Crybb1
Siglech
Cd9
Hexb
Trem2
P2ry12
Sall3
Selplg
Olfml3
Gpr84
Ms4a7
Lyz2
Cd74
Apoe
Pf4
Ms4a6c
Tgfbi
H2-Aa
Pla2g7
Clec12a
Mrc1
-2
0
2
4
Log2(FC) (Epiplexus MΦ vs. Stromal MΦ)

C


Sall1
Serpine2
Slc2a5
P2ry12
Ms4a7
Clec12a
H2-Aa
Mrc1

A **Primitive CSF system**

Future lateral ventricles | Future third ventricle | Future fourth ventricle | Macrophage

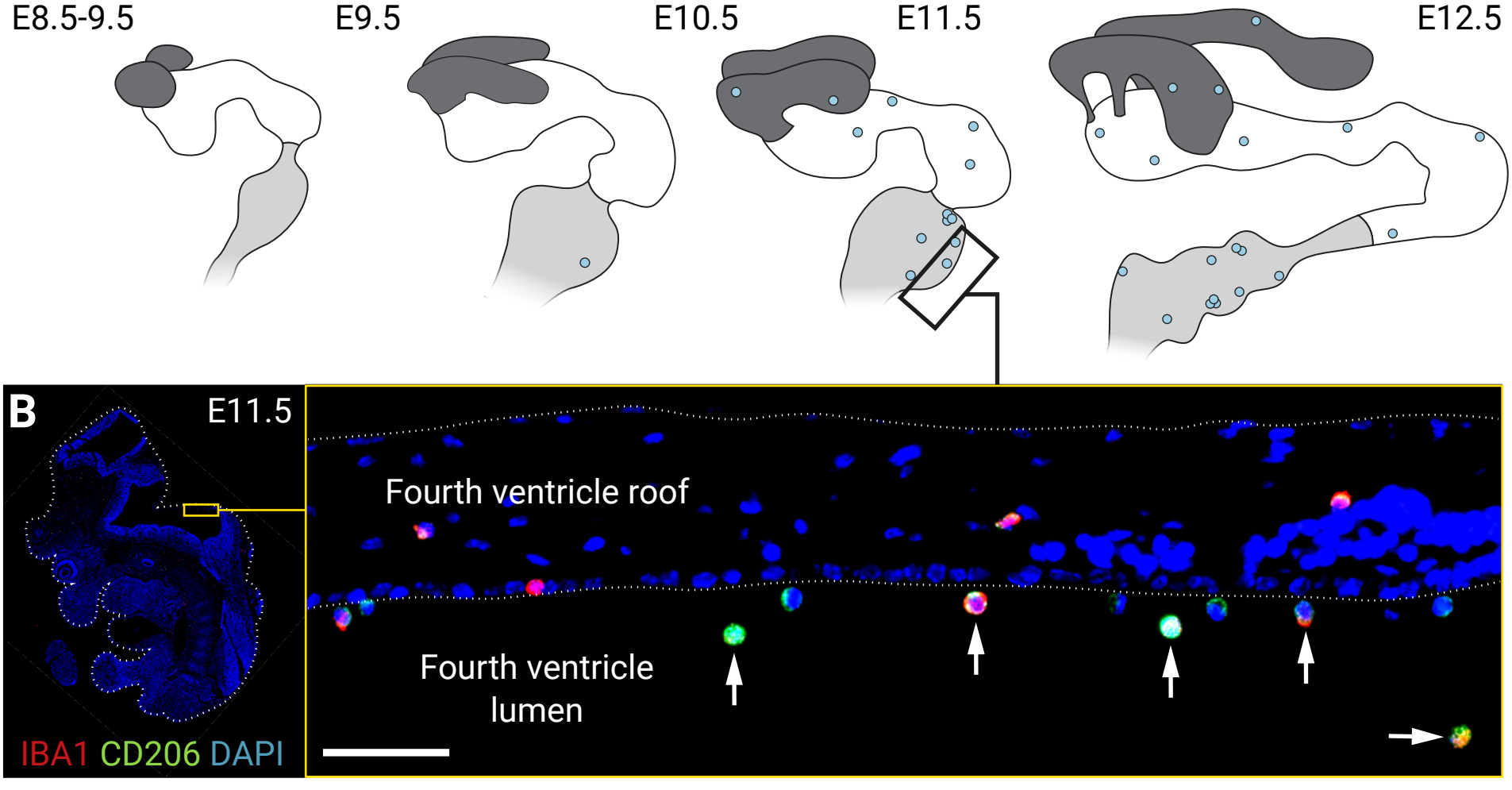
Neural tube closure → E8.5-9.5

First macrophages in cephalic mesenchyme and CNS parenchyma → E9.5

First CSF macrophages → E10.5

Choroid plexus development begins → E11.5

Stromal and epiplexus macrophages present in ChP of 4th/lateral ventricles → E12.5



B E11.5

IBA1 CD206 DAPI

Fourth ventricle roof

Fourth ventricle lumen

C **Choroid plexus cross-section**