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Brain macrophages in development, homeostasis and disease

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Abstract

Microglia are parenchymal macrophages of the central nervous system (CNS); as professional phagocytes they are important for maintenance of the brain's physiology. These cells are generated through primitive hematopoiesis in the yolk sac and migrate into the brain rudiment after establishment of embryonic circulation. Thereafter, microglia develop in a stepwise fashion, reaching complete maturity after birth. In the CNS, microglia self-renew without input from blood monocytes. Recent RNA-seq studies have defined a molecular signature for microglia under homeostasis. During disease, microglia undergo remarkable phenotypic changes, which reflect the acquisition of specialized functions tailored to the pathological context. In addition to microglia, the brain-border regions host populations of extra-parenchymal macrophages with disparate origins and phenotypes that have recently been delineated. In this review we outline recent findings that provide a deeper understanding of both parenchymal microglia and extra-parenchymal brain macrophages in homeostasis and during disease.

1. Ontogeny and phenotypic maturation of microglia

1.1 Microglia stem from yolk sac-derived myeloid progenitors

Microglia, the parenchymal macrophages of the CNS, originate from cKit^{+}CD31^{+}CD41^{+}CD45^{lo} erythromyeloid precursor cells (EMPs) generated during embryonic primitive hematopoiesis (1, 2). EMPs bud from clusters of Tie2^{+}CD34^{+}Flk1^{+} hemogenic endothelial cells in the yolk sac (YS) as early as E7.5, and provide the first cohort of macrophages and erythrocytes to the developing embryo (3–5). EMP-derived macrophage precursors (CD45^{+}CD115^{+}F4/80^{+}CX3CR1^{+}) appear in the blood around E8.5, spread via the embryonic circulation and seed all the tissues (including the CNS), wherein they complete the differentiation process (3, 6, 7). EMP commitment towards macrophages is supported by a combination of transcription factors, especially Runx1, PU.1 and IRF8 (4, 6, 8–10). Notably, microglia do not require the transcription factor Myb (11), which is necessary for the bone marrow hematopoiesis (12). YS-derived EMPs also migrate into the fetal liver, thus giving rise to the transient-definitive hematopoiesis. During the third week of gestation, EMPs in the liver are progressively outcompeted by hematopoietic stem cells (HSCs), which eventually establish definitive hematopoiesis in the bone marrow (13). Microglia first appear in the neuroepithelium at E9.5 (6, 8) (Figure 1). However, invasion of

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the embryonic CNS ends around E14.5, after the formation of the blood-brain barrier (BBB) (14). Thereafter, microglia spread throughout the CNS parenchyma and undertake a stepwise differentiation process, reaching a final maturation state around the second postnatal week (15, 16).

The concept that mouse microglia are solely derived from yolk sac EMPs dominates the field and is currently supported by several lines of evidence (3, 6, 17). However, a recent publication suggested that about 20-25% of brain microglia stem from Hoxb8-positive fetal liver-derived monocytes (FL-Mo), arising from fetal liver hematopoiesis around E12 (18). Thus, FL-Mo would enter the embryonic CNS and acquire phenotype and functions barely distinguishable from YS-derived microglia. Albeit highly provocative, this model might explain previous findings that contradict a sole YS origin for microglia. First, a transient increase in Ly6C^{+} monocytes was noted in the embryonic brain between E12 and E14 (19). Second, early depletion of YS-derived macrophages prevents the seeding of microglia in the brain rudiment at E10.5 and enhances the recruitment of monocytes at E14.5. Then, a microglial population reappears during the late gestational period, when EMPs are no longer present (19, 20). Third, the *S100a4*^{Cre} line, supposedly specific for monocytes, engenders fluorescent labeling in roughly 20% of embryonic microglia, and this population is maintained in the adult brain (19, 21). Further studies are needed to determine whether multiple hematopoietic waves contribute to microglia in mice. Current fate-mapping tools may not accurately resolve partially overlapping populations such as YS-derived macrophages and FL-derived monocytes. In the near future, techniques involving photoinducible labeling, such as NICHE-seq (22), might be exploited to track the fate of macrophage-progenitors *in vivo*.

1.2 Brain cytokines are critical for microglial survival and phenotype

Microglial survival is dependent on the CSF1R ligands CSF1 and IL34 (23–27). In the brain, IL34 is produced by neurons, whereas CSF1 is produced by both neurons (26, 28, 29) and microglia themselves (15). Interestingly, these cytokines exhibit non-overlapping expression patterns. CSF1 is highly expressed in the cerebellum, corpus callosum and spinal cord, whereas IL34 is more abundant in the neocortex, olfactory bulb, striatum and hippocampus (25–27). Genetic ablation of either CSF1 (24, 26) or IL34 (25, 27) results in a partial reduction of microglia, estimated to be about 30% and 50%, respectively. Furthermore, it has recently been shown that deletion of CSF1 in the neuroectodermal lineage causes specific depletion of cerebellar microglia (30). At present, it is not known whether CSF1-dependent and IL34-dependent microglia differ in terms of phenotype and function.

TGFβ is another critical factor for microglial homeostasis. In the brain, TGFβ is produced by microglia, astrocytes and neurons (31–33), whereas TGFβR1 and TGFβR2 are chiefly expressed in microglia (32–34). TGFβ knock-out mice exhibit a deficit of microglia development (34). Likewise, ablation of TGFβR2 in microglia using either the *Sall1*^{CreER} or the *Cx3cr1*^{CreER} conditional deletion systems disrupts the homeostatic morphology and phenotype of microglia (35–37). Mice with TGFβR2-deficient microglia were also shown to develop white matter pathology and a progressive limb paralysis during adulthood (36). Similarly, mice deficient for Lrrc33, a critical molecule for TGFβ signaling in macrophages,

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develop microglia alterations, progressive paralysis, and die prematurely (38, 39). At present, which component(s) of the SMAD transcriptional machinery are crucial for TGFβ signaling in microglia is still not clear.

1.3 Microglia are long-lived cells with slow turnover

Several independent studies have shown that blood monocytes do not contribute to microglial turnover under homeostasis (3, 40, 41). However, monocytes can infiltrate the CNS and generate monocyte-derived macrophages in the presence of BBB damage (42–44). At steady state, microglia self-renew through a slow but constant process of apoptosis and cell division in a stochastic manner (45–48). By contrast, microglia undergo clonal expansion under pathological conditions (47, 48). Microglia are rather long-lived cells, with a turnover rate of a few months that varies depending on the brain region they occupy. For example, the median lifespan of mouse cortical microglia is about 15 months (48). One study attempted to determine microglial turnover in the human brain and, although limited to only two subjects, found that human microglia proliferate at a very slow rate, with 0.08% cells being replaced per day. Using a mathematical model, the average lifespan of human microglia was estimated to be about 4.2 years, although some cells may be older than 20 years. This means that the entire microglial population is probably renewed multiple times during a human life (49).

1.4 Competition between resident microglia and monocytes for tissue niches

Depletion of microglia, either by injection of diphtheria toxin into mice expressing diphtheria toxin-receptor (DTR) in macrophages or via chronic administration of CSF1R inhibitors, has provided deep insight into the dynamics of microglia turnover. Following depletion, microglia rapidly proliferate and repopulate the CNS in a few days (50–52). Determining the origin of repopulating microglia has been an active field of research for the past few years. Multiple studies determined that virtually all repopulating microglia originate from a few microglial cells that survive the depletion period, whereas blood monocytes do not contribute to repopulation, at least in the absence of BBB disruption due to irradiation or myeloablative chemotherapy (35, 53, 54). At present, the factors promoting microglia repopulation are obscure. However, it has been shown that deletion of either IL-1R1 or IKKβ (upstream kinase of the NF-kB cascade) in microglia significantly delays the repopulation (52, 54). In stark contrast to these studies, a recent work using the *Cx3cr1*^{CreE}*R26-DTR* mice concluded that Ly6C^{hi} monocytes do engraft the brain parenchyma in the absence of head irradiation. Monocyte-derived microglia were identified as F4/80^{hi}Clec12a^{+}, whereas resident microglia were F4/80^{lo}Clec12a^{-} (55). Similarly, another group used *Cx3cr1*^{CreER}*Csf1r*$^{flox/flox}$ mice, which yielded partial depletion of microglia accompanied by monocyte recruitment into the CNS (56). It was also shown that irradiation-free transplantation of wild-type bone marrow into CSF1R-deficient pups (devoid of microglia) generates a massive invasion of donor-derived monocytes into the host CNS (57). Moreover, these studies have shown that resident and monocyte-derived microglia encompass phenotypically distinct populations. For example, unlike resident microglia, monocyte-derived counterparts do not express the transcriptional regulator Sall1 (35, 55, 57, 58). ATAC-sequencing revealed that certain loci typically open in resident microglia (like *Zfp691* (59, 60)), are transcriptionally inaccessible in monocyte-derived microglia (58).

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Conversely, monocyte-derived microglia express high levels of various immune-related genes (like MHC-II chains, *Lyz2, Clec12a, Ms4a7, ApoE, Cybb*) that are typically silenced in resident microglia at steady state (55, 57, 58). Together, these data indicate that microglia and monocytes may contend for the colonization of empty niches in the brain. Under naïve conditions, microglia greatly outcompete monocytes, perhaps by being more suited to the brain environment, or simply because their homing into the CNS occurs during primitive hematopoiesis, long before adult hematopoiesis is established in bone marrow. Nevertheless, under conditions of microglia deficiency, monocytes may gain access to the CNS and differentiate into macrophages that partially resemble microglia (61).

1.5 Both ontogeny and environment sculpt the molecular fingerprint of microglia

Local environmental cues are critical for the final maturation and specialization of tissue-resident macrophages, including microglia (7, 62–66). Indeed, acutely isolated microglia lose their molecular identity within a few hours upon exposure to cell culture conditions (60, 67). Conversely, cultured microglia transplanted back into the mouse brain reacquired their original phenotype in about two weeks (57, 67). Similarly, transplantation of iPSC-derived macrophages into the post-natal mouse brain generated microglia-like cells fully integrated within the host tissue (68, 69). Overall, the brain environment is critical for the maturation and maintenance of the microglial phenotype. Nevertheless, the failure of monocytes to acquire a complete microglial signature suggests that origin from the yolk sac or bone marrow may imprint different repertoires of poised enhancers. For example, it has been shown that both miRNAs (70) and HDAC enzymes (71) critically shape microglia development during the embryonic stage, but not after birth. Altogether, we can hypothesize that yolk sac ontogeny dictates the epigenetic landscape in microglial progenitors, whereas the transcriptional signature is locally instructed within the CNS environment (57).

2. Microglial phenotypes during homeostasis and disease

2.1 Developmental and regional heterogeneity of microglia

Bulk RNAseq studies identified a number microglia-specific genes, like *Crybb1, Fcrls, Gpr34, Gpr84, Hexb, Olfml3, P2ry12, P2yr13, Rnase4, Sall1, Siglech, Slc2a5, and Tmem119* (16, 32, 34, 59, 72). Nevertheless, caution should be used because evolving technologies for multidimensional analysis and deep sequencing always reveal previously unappreciated subpopulations. For example, *Siglech* is also a marker for plasmacytoid dendritic cells (pDC) (73). Although pDCs represent a minor population of meningeal DCs under homeostasis, encompassing about 1.5% of the total MHC-II^{+} cells of the brain (74, 75), their number expands significantly during disease (76). Moreover, recent studies showed that *Fcrls* is broadly expressed in multiple brain macrophage subsets (76, 77), whereas *Sall1* is apparently expressed in a population of macrophages within the apical choroid plexus epithelium (77).

More recently, single-cell RNAseq enabled more in-depth characterization of the transcriptional landscape in microglia at different stages of development. Embryonic microglia are enriched for various lysosomal enzymes, *ApoE* and *Ms4a7* (78, 79). By contrast, early postnatal microglia abundantly expressed *Igf1*, *Spp1*, *Gpnmb*, and *Clec7a*.

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Interestingly, Spp1^{+}Gpnmb^{+}Clec7a^{+} postnatal microglia were primarily found within heavily myelinated regions like the corpus callosum and cerebellum (78, 80). Of note, these studies consistently found a cluster of microglia particularly enriched for immediate early genes like *Fos, Jun* and *Egr1* (77–80). It was, however, acknowledged that this cluster may have been artificially generated due to microglia activation during sample preparation (77, 80, 81).

One month after birth mouse microglia are phenotypically mature with transcriptomes prominently enriched for homeostatic genes, such as *Tmem119, P2ry12, Slc2a5, Selplg, Cst3, Sparc, Tgfbr, Malat1* and others (78–80). Nonetheless, a region-dependent heterogeneity of microglia can be appreciated. For example, one study showed that microglia may vary phenotypically, depending on their topological distribution within the CNS (82). In particular, cerebellar microglia appeared skewed towards a more "immune alerted" and "metabolically demanding" phenotype, possibly because of the higher content of white matter as compared to other brain regions. By contrast, microglia in the cortex and striatum appeared in a more "quiescent" state, while hippocampal microglia had an intermediate phenotype. Another report identified transcriptomic heterogeneity in microglia selectively isolated from the cortex, nucleus accumbens, ventral tegmental area and substantia nigra (83). Ingenuity pathway analysis identified the highest variability in pathways for vesicle release, mitochondrial function, cell metabolism, oxidative stress, lysosomal activity and transport of metal ions. More recently, distinct patterns of gene-expression and epigenetic signatures were identified in cerebellar and striatal microglia. In particular, cerebellar microglia were highly enriched for genes linked to phagocytosis of apoptotic cells, while striatal microglia were more enriched for genes involved in immunological surveillance (84).

Lastly, molecular heterogeneity was recently described in human microglia. A mass cytometry study identified multiple region-specific subsets of microglia from postmortem brains (85). Consistently, single-cell RNAseq of microglia from healthy human brains formed multiple clusters with varying enrichment for *Tmem119, Cx3cr1, P2ry12, Slc2a5, Cst3*, *Ccl2* and *Ccl4* (79). Understanding the functional implications of such microglial phenotypes will be an important challenge for the years to come.

2.2 The DAM signature during pathology

Broad changes in the transcriptomic profile of microglia have been found in mouse models of amyloid pathology (86–88), Tau pathology (89), and Experimental Autoimmune Encephalomyelitis (EAE) (74, 76, 88). Altogether, pathological conditions cause downregulation of the microglial homeostatic genes (including *Tmem119, P2ry12, Selplg, Cx3cr1, Tgfbr1, Sall1*), whereas other genes are upregulated. For example, *Trem2*, *Tyrobp*, and *ApoE* were consistently found overexpressed in microglia in different neurodegeneration mouse models (86–92). Moreover, microglia exhibited *ApoE* upregulation during EAE (76, 88), and in the cuprizone model of toxic demyelination (79). The exact functions of TREM2 and ApoE during brain diseases are still controversial, and this topic has already been addressed elsewhere (93–96). Interestingly, independent studies identified a conserved molecular signature of microglia in models of amyloid pathology and

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neurodegeneration (86–88). This specific microglial phenotype has been termed “Disease-Associated Microglia” (DAM) (86).

In mouse, the DAM signature is characterized by higher expression of genes involved in lysosomal functions (Cst7, Ctsb/d), phagocytosis (Axl), antigen presentation (H2-Aa, H2-Ab), lipid transport (Lpl, Apoe), matrix remodeling (Spp1, Gpnmb), complement binding (CD11c), anti-microbial activity (Lyz2, Dectin-1), immune modulation (Lilrb4), and cell survival (Csf1, Igf1) (74, 77, 86–88) (Figure 2).

Cystatin F (Cst7) is a lysosomal cysteine protease inhibitor, which targets the lysosomal enzyme cathepsin C (97). Inhibition of Cst7 expression correlates with reduced amyloid pathology (98), indicating that Cst7 may reduce the capacity of microglia to degrade Aβ. Alongside, DAM signature is also characterized by higher expression of some cathepsins, especially Ctsb and Ctsd (86). Cathepsins are cysteine proteases important for lysosomal degradation of aggregated proteins (99). Additionally, cathepsins can be secreted, and therefore may play a role in cell migration (100).

Axl and Mertk belong to a family of TAM tyrosine kinases receptors mainly involved in the phagocytosis of dead cells (101). Axl is upregulated in microglia during neurodegeneration and neuroinflammatory diseases (86–88, 102), whereas Mertk is downregulated (74, 86, 88). Microglia lacking Axl and Mertk exhibit deficient phagocytosis of apoptotic cells and display reduced migration towards laser-induced injury (102, 103). In the EAE model, *Axl*-deficient mice show a more severe pathology and fewer macrophages infiltrating the spinal cord (104). Whether Axl or Mertk are directly involved in the uptake of protein aggregates (such as Aβ and α-synuclein) remains obscure.

Secreted Phosphoprotein-1 (Spp1, Osteopontin), is one of the most upregulated genes in the DAM signature (77, 86–88) and significantly increased levels of Spp1 were found in the CSF of AD and FTD patients (105, 106). In the periphery, Spp1 is highly expressed by both osteoblasts and osteoclasts and is important for the bone mineralization (107, 108). Spp1 is also secreted by different leukocytes including Th1 cells, macrophages and DCs (109). Spp1 induces IL12 production in DCs, thus promoting type-I immunity (110, 111). Moreover, Spp1 was shown to improve survival of autoreactive T-cells in EAE (112). However, it was also suggested that Spp1 regulates inflammatory reactions (113), for example via inhibition of NO production in macrophages (114). Spp1 is also known to bind CD44 (110, 115), which, in the brain, is primarily expressed by astrocytes (32, 33, 116), while the expression in microglia is negligible (117). Spp1 and CD44 may then form a communication axis between microglia and astrocytes under neurodegeneration. Additionally, secreted Spp1 represents a substrate for the activation of metalloproteinases (118), suggesting that it may play some role in microglia migration towards the injury site. Further studies on conditional Spp1-deficient mice are required to better understand the function of this protein in microglia. Similar to Spp1, Osteoactivin (Gpnmb) represents another possible ligand for CD44 (119). It was indeed suggested that Gpnmb may dampen astrocyte activation via CD44 signaling (120).

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Clec7a (Dectin-1) is a C-type lectin serving as pattern-recognition receptor against fungi and bacteria (121). Clec7a contains an ITAM motif promoting a Syk-dependent signaling, which elicits an anti-microbial response in macrophages (122, 123). Albeit not expressed under homeostasis, Clec7a^{+} microglia were found in proximity of amyloid plaques (86, 88), as well as in mice with microglia-specific BRAF mutation, which causes microgliosis and late-onset neurodegeneration (124). *In vitro*, microglial metabolic activity is boosted by zymosan, which is a known ligand for Clec7a (125). Possibly, Clec7a may help mount the immune-activation state of plaque-associated microglia. Moreover, Aβ is known to induce microglia activation via various pattern-recognition receptors (126). It remains a question whether Clec7a is involved in a similar mechanism.

CD11c (*Itgax*), a prototypical DC marker, has repeatedly been found upregulated in activated microglia and represents one of the most consistent DAM signature genes (74, 77, 86, 88). CD11c and CD18 form the complement-receptor 4, which is important for the engulfment and fragmentation of complement-opsonized particles (127). It is then tempting to speculate that CD11c in plaque-associated microglia may help recognize or phagocytose Aβ aggregates. A conditional knock-out mouse model is needed to better investigate the role of CD11c in microglia under pathology.

Leukocyte immunoglobulin-like receptor B4 (*Lilrb4*) belongs to a family of ITIM-bearing inhibitory receptors widely expressed in different leukocytes (128). At present, the ligand as well as the exact function of mouse Lilrb4 is unknown. A recent study showed that conditional ablation of Lilrb4 exacerbates steatosis and systemic inflammation in mice under hyper-fat diet (129), suggesting that this receptor may play important immunomodulatory functions. Whether Lilrb4 could restrain the microglia activation state under brain pathology remains hypothetical.

Lastly, the DAM signature shows an enrichment for *Csf1* and *Igf1*. Interestingly, both genes are highly expressed in microglia at the early stages of brain development (15, 80, 130, 131). Csf1 is important for microglial survival and proliferation (51, 132), whereas microglia-derived Igf1 was shown to support the survival of newborn neurons (130). Csf1 could act on microglia in an autocrine/paracrine manner, thus promoting microglia proliferation/survival within the plaque-surrounding environment. RNAseq data indicate that expression of Igf1r in microglia is negligible, but detectable in neurons (32, 33). Microglia-derived Igf1 could then provide neurotrophic support to the neighboring neurons, thus protecting against Aβ cytotoxicity.

2.3 Similarities and discrepancies between mouse and human microglia

Our understanding of the molecular properties of microglia relies chiefly on mouse models. At present, only a few pioneering works have delineated the transcriptomic profiles of microglia from healthy subjects, as well as from patients with Alzheimer's Disease (AD) and multiple sclerosis (MS) (60, 79, 116, 133). A seminal study showed that the transcriptomes of both mouse and human microglia are characterized by a dominant PU.1-dependent signature and about 50% of the microglia-specific genes (such as *Cx3cr1, Tmem119, P2ry12, Trem2* and *Sall1*) are similarly expressed in both species. Using a cutoff of 10-fold, 2.5% of the transcripts were highly enriched in human microglia (like *C3, SPP1*,

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and *APOE*) and 1.9% appeared specific for mouse microglia (like *Hexb, Sparc*, and *Sall3*). Overall, these data indicate a substantial overlap between mouse and human microglia at the molecular level (60). Similar findings were reported in a following study that, however, highlighted increasing molecular disparity between mouse and human microglia during ageing (134). A mass cytometry study showed that human microglia express high levels of TMEM119 and P2RY12, which are absent in blood myeloid cells. Human microglia are also positive for EMR1 (F4/80), TREM2, CX3CR1, CD64 and CD115, whereas expression of CD45, CD44, CCR2, CD206 and CD163 is either low or negligible. This repertoire of surface markers closely resembles that of mouse microglia. However, unlike their mouse counterparts in the steady state, human microglia express CD11c, MHC-II, and relatively low levels of CD11b (85).

In AD patients, microglia upregulate *CD74, HLA-DR, APOE, TREM2, C1Q* and *CD14*. Interestingly, transcriptomic changes in microglia seemed to correlate with the severity of both amyloid and tau pathology (133). Given the difficulties of working with human brain samples, it has been suggested that iPSCs may be a powerful tool for modeling human microglia during brain diseases (68, 69, 135, 136). For example, a recent study showed that following transplantation into the brains of mice with amyloid pathology, iPSC-derived human microglia efficiently migrated towards amyloid plaques and, like their murine counterparts, up-regulated *APOE, HLA-DR, LGALS3, MS4A7, ITGAX*, and *TREM2*. Of note, some DAM signature genes like *TYROBP, CST7, CLEC7A* and *CSF1* were not significantly altered in iPSC-derived human microglia (137). This suggests that mouse and human microglia mount similar immunological responses against amyloid pathology *in vivo*; however, certain pathways may not be conserved between species. Our own data support this view, as we detected a prominent IRF8-dependent signature in human microglia from AD patients, but not in mouse models (138). Further studies are required to better understand similarities and differences in the DAM signatures of human and mouse microglia.

3. Shared and distinct properties of microglia and Border Associated Macrophages (BAMs)

3.1 Ontogeny and phenotype of BAMs

Microglia are not the only brain-resident macrophages; indeed, populations of extra-parenchymal macrophage patrol the brain-blood interface in both mice and humans. Perivascular macrophages (pvMPs) are primarily found in the perivascular Virchow-Robin spaces of the cortical blood vessels. Meningeal macrophages (mMPs) are located within the meningeal membranes, either on the pia mater or within the dura. Lastly, choroid plexus macrophages (cpMPs) lie beneath the epithelial cell layer of the choroid plexus (139). These extra-parenchymal brain macrophages are collectively referred to as border associated macrophages (BAMs) (74). Although microglia and BAMs share the expression of several phenotypic markers (including Iba1, CD11b, CX3CR1, CD64, Mertk, CD115, and others), transcriptome studies identified a repertoire of molecules that are specifically expressed by each population (Figure 3). Like microglia, BAMs are dependent on CSF1R signaling for their survival (74, 77). However, it is still unknown whether anatomically distinct BAMs

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subsets are differentially dependent on CSF1 and IL34. Interestingly, genetic deletion of the super-enhancer *fms-intronic regulatory element* (FIRE), which is critical for the expression of CSF1R in YS-derived macrophages, causes complete depletion of microglia, but leaves pvMPs and mMPs seemingly unaffected. This may suggest that transcriptional and epigenetic mechanisms differentially regulate CSF1R in microglia and extra-parenchymal macrophages (140).

It has long been thought that BAMs are derived from and constantly replaced by BM-derived monocytes. However, recent fate-mapping studies revealed that, like microglia, BAMs are generated by YS-derived progenitors. Embryonic-derived pvMPs and mMPs are long-lived cells, whereas cpMPs are rapidly replaced by BM-derived monocytes soon after birth (141). Of note, a recent paper described a population of BAMs localized on the apical choroid plexus epithelium (previously identified as epiplexus Kolmer's cells) that share key features of microglia. These cells are embryonic derived, express Sall1 and can self-renew with no obvious input from the periphery. By contrast, stromal cpMPs are negative for Sall1 and undergo constant monocyte-mediated turnover (77). At present, it is unclear whether YS-derived myeloid progenitors are already committed to become either microglia or BAMs during the embryonic development, or whether the two differentiation pathways are locally instructed by environmental stimuli. The first case predicts the existence of a common myeloid progenitor (possibly downstream of the EMP stage) that generates two separate lineages. Alternatively, we may hypothesize that the local environment of the blood-brain interface guides the differentiation of embryonic macrophages towards a BAM phenotype, whereas the parenchymal environment supports the differentiation into microglia (Figure 4). Future studies will hopefully shed some light on this outstanding question.

3.2 Antigen-presentation capacity of BAMs and microglia during neuroinflammatory diseases

The composition of BAMs during neuroinflammatory disease has recently been investigated by single-cell RNAseq and multi-dimensional immunophenotyping techniques. During EAE, for example, expression of several activation markers (MHC-II, CD74, CD44, Sca-1, and CD11c) was increased, whereas the BAM homeostatic markers Lyve-1, Fcrls, and CD206 were downregulated. Interestingly, expression of other markers like Ms4a7, CD38, CD169 and CD86 remained stable during disease (74, 76). Meningeal myeloid cells have often been implicated in the re-activation of auto-aggressive T-cells during EAE (142–145). Nonetheless, experimental proof that BAMs license T-cell entry into the CNS is lacking. A landmark publication used intravital two-photon imaging to illustrate the path of autoreactive T-cells in a rat model of transfer EAE (146). Both MBP and MOG reactive T-cells enter the CNS by crawling along the leptomeninges, where they randomly encounter mMPs. Given that the leptomeninges represent a main gateway for infiltrating T-cells, mMPs probably constitute the first line of antigen-presenting cells (APCs) at the brain/blood interface. TCR engagement by meningeal APCs induces nuclear translocation of NFAT in antigen-competent T-cells, thereby eliciting their encephalitogenic potential (146). Intrathecal administration of blocking antibody for LFA1/VLA4 integrins augmented the number of MBP-reactive T-cells in the CSF, but dramatically reduced their infiltration into the underlying parenchyma (146). Consistent with this, in a rat model of gray matter

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inflammation, the number of synuclein-reactive T-cells was diminished in both the parenchyma and meninges after intrathecal injection of anti-MHC-II antibody. In addition, this treatment significantly attenuated expression of inflammatory cytokines (IFNγ and IL17) as well as the EAE clinical score (147). Altogether, these findings indicate that the meningeal myeloid compartment represents a critical checkpoint for the re-stimulation of auto-reactive T-cells before their invasion of the CNS parenchyma. To more precisely interrogate the antigen-presentation capacity of microglia and BAMs during EAE, three independent groups studied the effect of MHC-II deletion in microglia using either the *Cx3cr1*^{CreER} or the *Sall1*^{CreER} inducible systems. EAE pathology was unaffected in mice with MHC-II deficient microglia in all three studies (75, 76, 148). By contrast, deletion of MHC-II using the CD11c^{Cre} line (which targets both DCs and activated macrophages) completely abolished the onset of paralysis, CNS infiltration and demyelination (76). Additional studies are now required to clarify whether meningeal DCs are uniquely competent APCs in the brain, or whether other BAMs also play a role in this context. Nevertheless, these data strongly indicate that microglia are probably dispensable for the reactivation of T-cells during EAE.

3.3 Microglia promote both tissue damage and repair during neuroinflammation

Under neuroinflammation, microglia might significantly contribute to tissue damage by releasing inflammatory cytokines. A mass cytometry study in EAE identified seven distinct microglial populations expressing variable levels of pro-inflammatory (mostly TNF-α, IL-6, GM-CSF) and anti-inflammatory (TGF-β and IL-10) cytokines (149). This may indicate that microglia both promote and help resolve neuroinflammation during EAE. Furthermore, a recent study based on two-photon intravital microscopy during EAE suggested that CNS phagocytes shift from a pro-inflammatory to a wound-healing phenotype, depending on the lesion context (150). A seminal study on *Cx3cr1*^{CreER}*Tak1*$^{flox/flox}$ mice showed that deletion of the NF-kB activator Tak1 in microglia remarkably ameliorated EAE pathology in mice (41), which implies that microglia can be gravely detrimental in neuroinflammatory conditions. However, like microglia, meningeal and perivascular macrophages are CX3CR1$^+$ and undergo very limited turnover (141). Therefore, the observed protective effect could partially stem from Tak1 deletion in BAMs, rather than microglia. In contrast, deletion of NF-kB negative regulators (like AHR or A20) in microglia was shown exacerbate neuroinflammation and EAE pathology (151, 152). Similarly, the Ubiquitin Specific Peptidase 18 (*USP18*) was shown to act as a negative regulator of the Stat1 pathway in white matter microglia, thus dampening the type-I interferon response (153). Therefore, microglia lacking *USP18* constitutively upregulate IFN-dependent genes resulting in white-matter pathology and behavioral defects (154).

Besides their pro-inflammatory function at the peak of the disease, microglia may play a critical role in the resolution of inflammation and tissue repair. Indeed, microglia were repeatedly shown to be important for the clearance of myelin debris and ensuing remyelination both in EAE (155) and toxic-induced demyelination models (156–160). This suggests that microglia can support the healing of the white matter after demyelination. Indeed, a recent study showed that a population of CD11c$^+$Clec7a$^+$Gpnmb$^+$ microglia promote remyelination via secretion of Igf1 during EAE (131). Additionally, microglia were

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shown to upregulate PD-L1 in the EAE model (74), whereas a microglial subset expressing Galectin-1 was found in post-mortem brain samples from MS patients (79). Both of these genes critically modulate the activation of CD8+ lymphocytes (161–164). It is then tempting to speculate that microglia could help dampen the T-cell mediated cytotoxic response during MS. Of note, microglia depletion approaches have generated conflicting results in the context of EAE, resulting in either beneficial, detrimental or no effects (165–167). We would hypothesize that non-selective depletion of both microglia and BAMs may produce unpredictable confounding effects.

Altogether, microglia may promote tissue damage during neuroinflammation via cytokine and ROS production. However, microglia are also critical for efficient scavenging of cellular debris and tissue regeneration. Environmental cues that selectively promote these functions are currently under investigation.

4. Conclusions

Brain macrophages encompass multiple populations characterized by different anatomical distribution, phenotype, ontogeny/turnover, and, very likely, different functions. Growing evidence suggests that molecular signature of microglia and BAMs is instructed by a combination of local environment and ontogeny. However, whether microglia and BAMs arise from a unique YS-derived progenitor or develop through independent pathways is still unknown (see also 168). Additionally, we just started to scratch the surface of the transcriptomic changes in brain macrophages during disease. For example, a number of independent works provided a list of candidate genes which identifies the DAM signature of plaque-associated microglia during amyloid pathology. Nevertheless, the exact function of these genes remains to be determined. This information may help us harness specific DAM genes to either reduce amyloid burden or improve viability of neighboring brain cells in Alzheimer Disease.

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Glossary

CNS	Central Nervous System
EMPs	Erythromyeloid Precursor Cells
YS	Yolk sac
HSCs	Hematopoietic Stem Cells
BBB	Blood-Brain Barrier
FL-Mo	Fetal Liver-derived Monocytes
DTR	Diphtheria Toxin-Receptor

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DAM	Disease-Associated Microglia
AD	Alzheimer Disease
MS	Multiple sclerosis
BAMs	Border Associated Macrophages
pvMPs	Perivascular macrophages
mMPs	Meningeal macrophages
cpMPs	Choroid plexus macrophages
EAE	Experimental Autoimmune Encephalomyelitis

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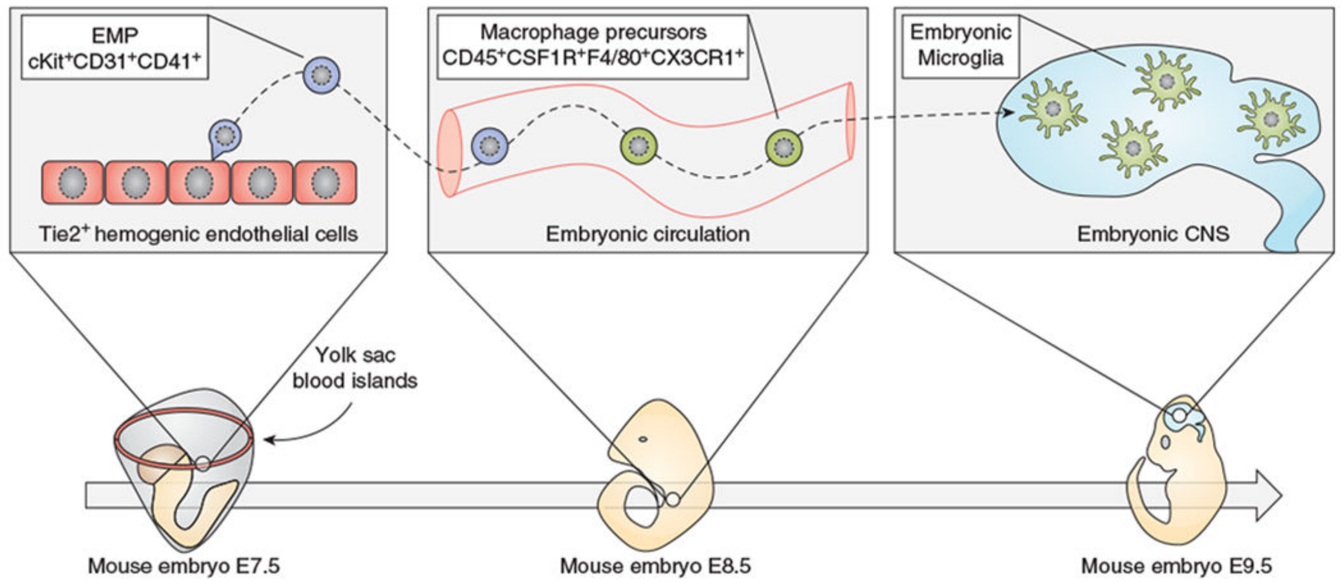

Figure 1:

Key steps in the development of embryonic microglia

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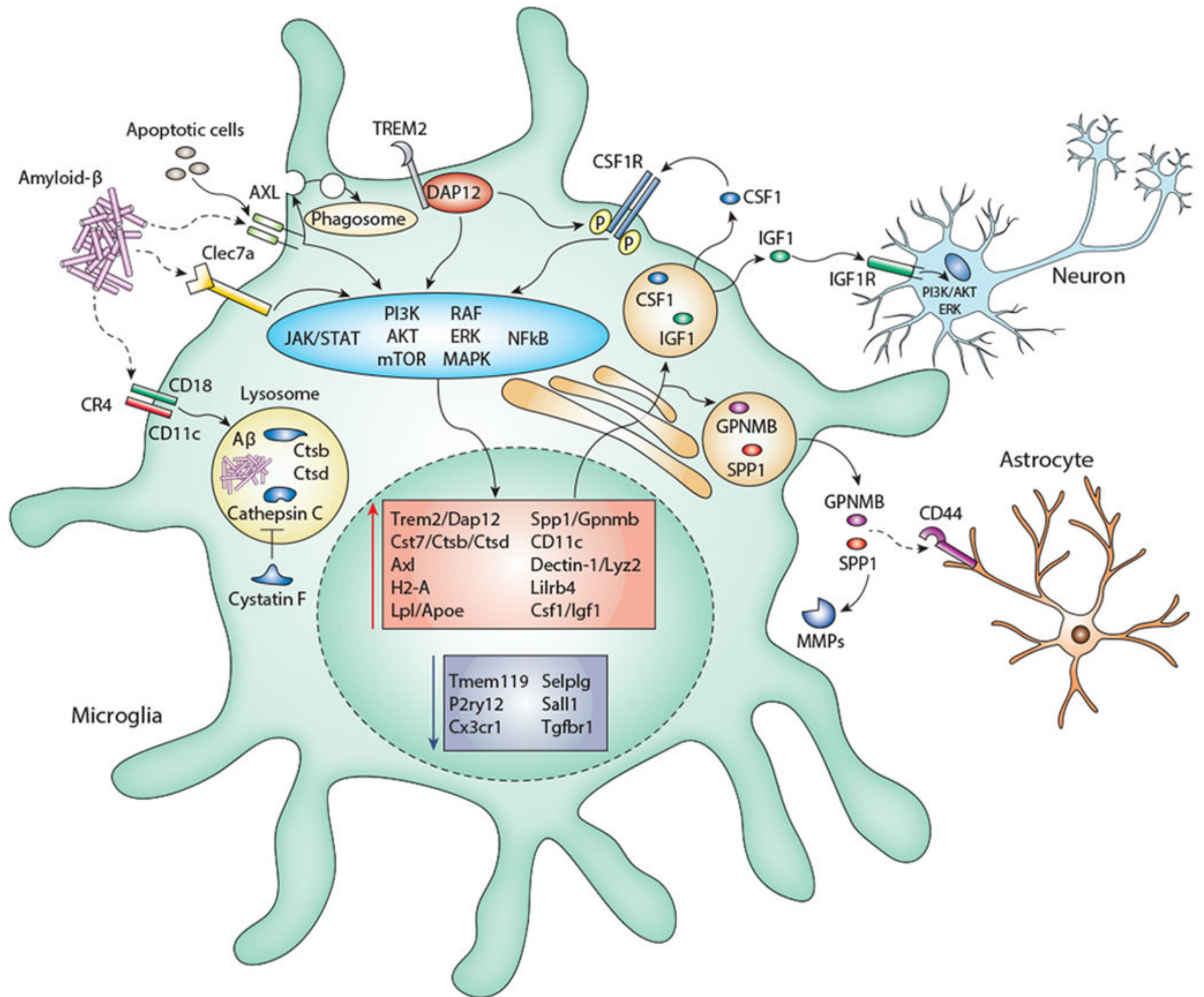

Figure 2:
Possible mechanisms involving the DAM signature genes.

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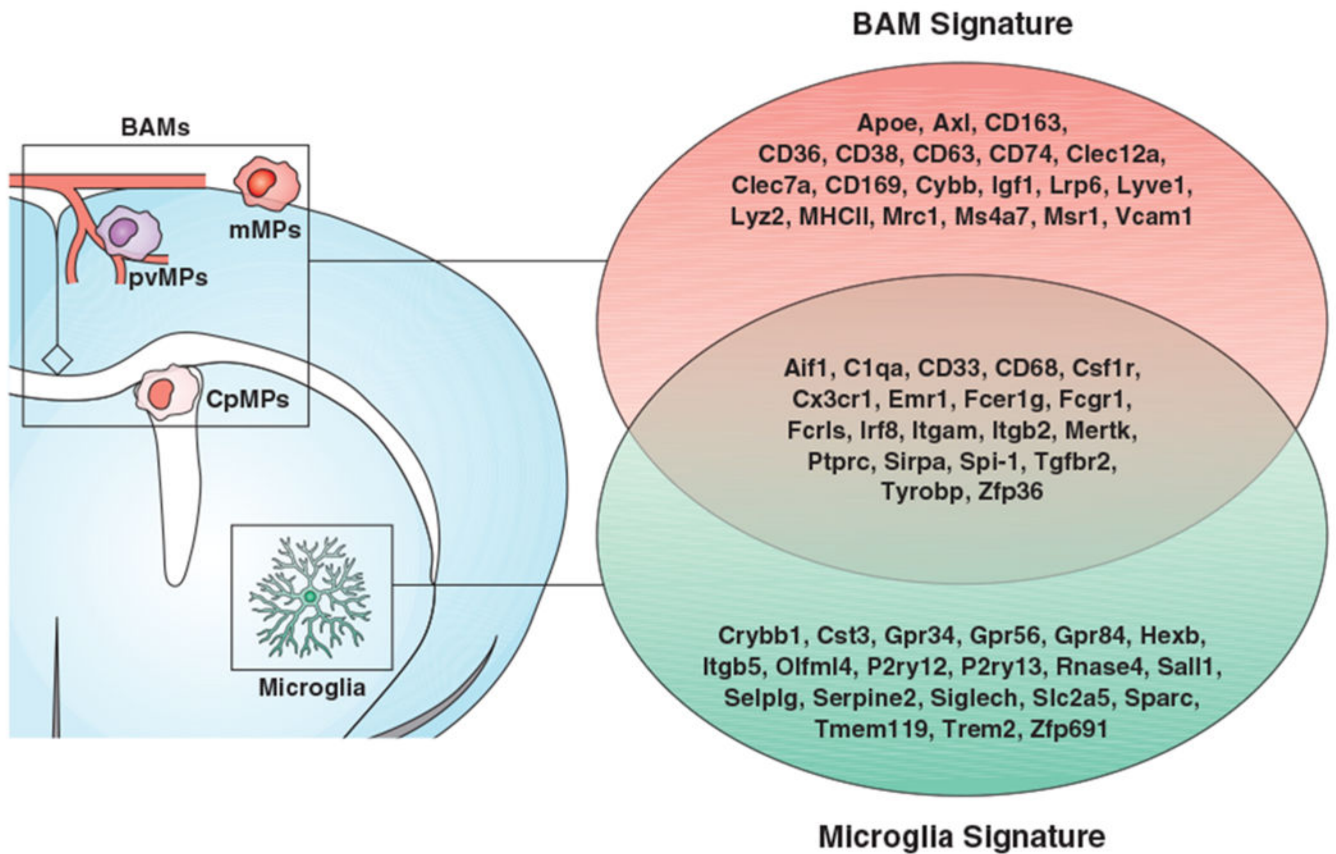

Figure 3:
Molecular signature of microglia and BAMs under homeostasis.

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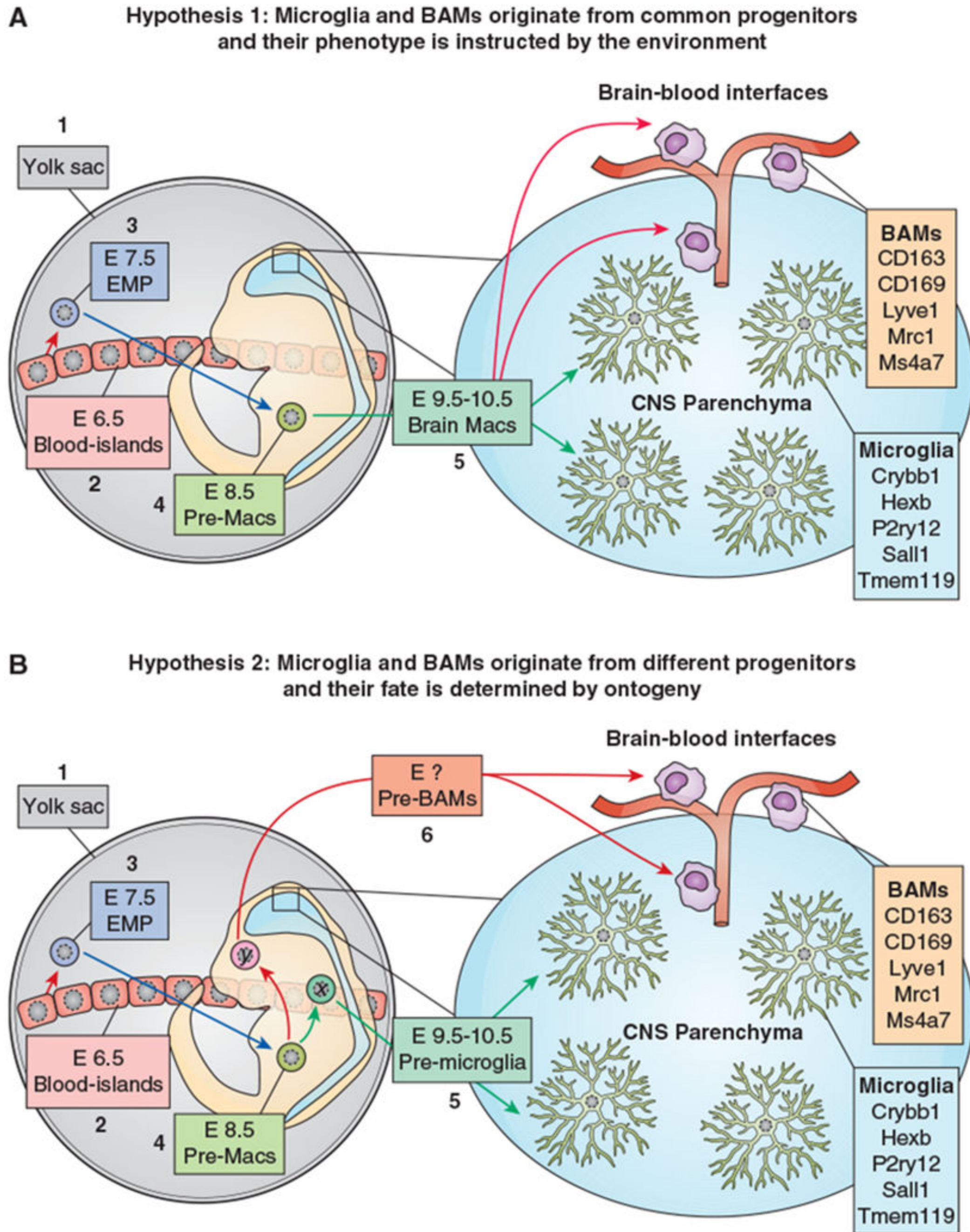

Figure 4:

Two alternative hypotheses for the origin of microglia and BAMs.

a) Hypothesis 1 - EMPs arise from YS-blood islands around E7.5 (1) and at E8.5 migrate into the embryo proper where they differentiate into macrophage precursors (pre-Macs) (2). These cells seed all embryonic tissues including the brain (3). Depending on the local environment, they differentiate into either microglia (4) or BAMs (5). In this model, microglia and BAMs originate from common progenitors and the local environment plays a

major role in determining their fate. The genes indicated in boxes 4 and 5 refer to transcriptional signatures identified in the adult mouse brain.

b) Hypothesis 2 - EMPs arise from YS-blood islands around E7.5 (1) and at E8.5 migrate into the embryo proper where they differentiate into macrophage precursors (pre-Macs) (2). These cells generate two separate lineages (x and y) giving rise to either pre-microglia (3) or pre-BAMs (4), which respectively colonize the CNS parenchyma and the brain-blood interfaces. The local environment dictates the final maturation into microglia (5) and BAMs (6). In this model, microglia and BAMs share a common ancestor cell (EMP), but eventually develop through distinct lineages. Therefore, a combination of ontogeny and environment is critical for the fate of both cell types. The genes indicated in boxes 5 and 6 refer to transcriptional signatures identified in the adult mouse brain.

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