ANNUAL REVIEWS

Annual Review of Genetics

Microglial Transcriptional Signatures in the Central Nervous System: Toward A Future of Unraveling Their Function in Health and Disease

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Annu. Rev. Genet. 2023. 57:65–86

First published as a Review in Advance on June 29, 2023

The *Annual Review of Genetics* is online at genet.annualreviews.org

https://doi.org/10.1146/annurev-genet-022223-093643

Keywords

microglia, microglial heterogeneity, RNA sequencing, transcriptomic signatures, central nervous system

Abstract

Microglia, the resident immune cells of the central nervous system (CNS), are primarily derived from the embryonic yolk sac and make their way to the CNS during early development. They play key physiological and immunological roles across the life span, throughout health, injury, and disease. Recent transcriptomic studies have identified gene transcript signatures expressed by microglia that may provide the foundation for unprecedented insights into their functions. Microglial gene expression signatures can help distinguish them from macrophage cell types to a reasonable degree of certainty, depending on the context. Microglial expression patterns further suggest a heterogeneous population comprised of many states that vary according to the spatiotemporal context. Microglial diversity is most

pronounced during development, when extensive CNS remodeling takes place, and following disease or injury. A next step of importance for the field will be to identify the functional roles performed by these various microglial states, with the perspective of targeting them therapeutically.

1. INTRODUCTION

Microglia, which are the resident innate immune cells of the central nervous system (CNS), are often described as CNS tissue-resident macrophages. Microglia predominantly develop from KIT^+ precursors generated in the embryonic yolk sac that migrate into the CNS (19, 36, 45, 57, 64). They are subsequently maintained, primarily through clonal expansion, within the CNS (42, 67). Macrophages—particularly tissue-resident macrophages such as those in the gut, alveolar macrophages in the lung, and Kupffer cells in the kidney—can also be derived from the embryonic yolk sac and fetal liver; to varying degrees, tissue-resident macrophages can also be replenished from circulating monocytes (45, 76). Despite their different ontologies, microglia and macrophage cell types often appear similar in phenotype, morphology, and function within the CNS, particularly in the context of disease or inflammatory conditions (45, 77). Recent work has identified genes that are highly upregulated in microglia, typically during the developmental steady state or in disease, although many of these transcripts are also found in other cell types, including monocytes and macrophages, particularly when exposed to a similar CNS environmental milieu (49, 77). As such, it is a current focus of the field to delineate between these cell types throughout development and across the life span, in health and disease, given the crucial involvement of microglia in numerous CNS processes. This may allow for either targeting blood and peripheral cells for CNS health or developing approaches to modulate cells in the CNS specifically, without affecting those in the periphery.

Phagocytosis: engulfment and intracellular clearance of cellular debris, cells, or parts of cells including synapses

Synaptic pruning: synapse elimination by microglia; can be normative (as during development) or related to pathology and can involve different mechanisms

Trogocytosis: nibbling or partial phagocytosis of cellular debris, cells, or parts of cells including synapses

Synaptic stripping: the physical disruption of the synapse by microglial processes intervening between pre- and post-synaptic elements

A previous widely held view of microglia positioned them as "quiescent" or "resting" under steady-state conditions. According to this now-rejected view, "activated" microglia exhibited detrimental production of proinflammatory cytokines and reactive species, as well as excessive phagocytosis, contributing to pathology during disease and injury. Microglia are now understood to exhibit many beneficial functions, including those beyond their immunological ones, across the life span (49, 58, 60, 66, 74). Microglia play regulatory and supportive roles for the other CNS cell types, including astrocytes, oligodendrocyte lineage cells, and neurons, and they also contribute to modulating physical brain structures, including blood vessels and the blood–brain barrier (BBB) (48, 60). Specifically, microglia support astrocyte differentiation, maturation, and function, including astrocytic control of (synaptic) glutamate and astrocytic reactivity during pathology (28). Furthermore, microglia can facilitate oligodendrocyte progenitor cell differentiation and oligodendrocyte myelination, phagocytosis of myelin debris, and myelin refinement (28, 48). With regard to neurons, microglia also contribute to neurogenesis and alterations of neuron numbers and produce factors that modulate neuronal migration and survival (59). Additionally, microglia physically refine neuronal circuits through dendritic spine formation and synaptic pruning, which can involve phagocytosis (i.e., engulfment and clearance of cellular debris, cells, or parts of cells) or trogocytosis (i.e., the nibbling of synapses). They also physically alter synaptic function and mediate plasticity through synaptic stripping (i.e., the physical disruption of the synapse by microglial processes) and by releasing factors such as brain-derived neurotropic factor and microRNAs that regulate synaptic function (70).

Given the importance of microglia throughout the life span in normative CNS functioning and pathology, as well as recovery, elucidating how to target microglia, in their heterogeneity, across

Table 1 Common methodologies for transcriptional and epigenetic assessment

Tool	Sample type	Analysis	Comments
Transcriptomics			
Microarray	Bulk or single cell (uncommon)	Gene expression	Requires specialized probes (limited number per assay)
RNA sequencing (RNA-seq)	Bulk or collections of cells	Global gene expression	Differential expression (common) or transcriptome analysis (e.g., splicing variants, long noncoding RNAs)
Single-cell RNA sequencing (scRNA-seq)	Single cell	Gene expression	Requires sample dissociation
Single-nuclei RNA sequencing (snRNA-seq)	Single cell; can be performed from frozen samples	Gene expression	Limited to nuclear transcripts (versus cytosolic and nuclear)
Massively parallel RNA single-cell sequencing (MARS-seq)	Single cell	Gene expression	High-throughput multiplexing; ideal for heterogenous samples
Epigenetics			
Chromatin immunoprecipitation sequencing (ChIP-seq)	Bulk or single cell	Transcription factor binding sites; histone modification	When used in combination with RNA-seq, can help understand gene regulatory networks
Methylated DNA immunoprecipitation sequencing (MeDIP-seq)	Bulk	DNA methylation	Typically represents repressed gene transcription; other methods exist for single-cell analysis
m^6A-methylated RNA immunoprecipitation with next-generation sequencing (MeRIP-seq)	Bulk	RNA methylation	Modifies RNA splicing, processing, translation, and degradation; other methods exist for single-cell analysis
Assay for transposase-accessible chromatin using sequencing (ATAC-seq)	Bulk or single cell	Chromatin accessibility	Discovery-based; often used in combination with RNA-seq

different contexts is critical. This is especially true when considering that many genes that confer risk for neurological diseases, including, for example, schizophrenia and Alzheimer's disease (AD), are enriched or preferentially expressed in microglia rather than in the whole brain or CNS (20). Therefore, tools that allow for the elucidation of microglial transcriptional and epigenetic changes (see common methodologies highlighted in **Table 1**) in health and disease states are a focus of the field. This review outlines the current understanding of microglial transcriptional signatures in relation to health and disease, from the perspective of addressing their function and designing strategies to target them therapeutically. We focus on recent studies highlighting transcriptional and epigenetic sequencing results, using microglia isolated from human and rodent (primarily mouse) CNS samples, across multiple stages of the life span in the steady state and in response to disease. We highlight similarities (and differences) in the defined microglial transcriptional signatures between time points, CNS regions, species, and disease conditions, as well as comment on potential functions of some of the associated transcripts that provide insights into possible future treatments, and discuss current field limitations.

2. MICROGLIAL TRANSCRIPTIONAL SIGNATURES AND THEIR RELATION TO HEALTH AND DISEASE

2.1. Developmental Programming

Microglia are considered CNS tissue-resident macrophages. Macrophages can be derived from (*a*) hematopoietic erythro-myeloid progenitors (EMPs), also termed primitive macrophages, in the yolk sac; (*b*) EMP-seeded monocytes in the fetal liver; or (*c*) blood monocytes derived from hematopoietic stem cells (30, 50). Most microglia are derived from the first wave of EMPs (30). A current view is that transcription factor (TF) profiles are conserved across early primitive macrophage populations (i.e., lineage-determining TFs) in development.

Next, exposure to the local microenvironment, in this case the CNS, is hypothesized to result in conditions that enhance some conserved lineage-determining TFs, as well as activate others (cell type–specific TFs), leading to the emergence of core microglial functions (20). An early synthesis (20), highlighting data from both mice and humans, described the following TFs as associated with primitive macrophages: AP-1, CEBPB, IRF8, KLFs, MAFs, MEFs, RUNX1, SMAD, and SPI/PU.1 (20). The following TFs were considered to be induced and/or activated in the brain: AP-1, EGR1, KLFs, MAFs, MEFs, SALL1, SMAD, and ZFP691 (20) (all genes and proteins are described in **Supplemental Table 1**).

Supplemental Material >

Developmental analysis in mice revealed clustering of CX3CR1^{+} microglia at different time points using population-level RNA sequencing (RNA-seq) (47). Matcovitch-Natan et al. (47) identified four developmental clusters: yolk sac [embryonic days (E)10.5, 11.5, 12.5, and 13.5 in the yolk sac], early microglia (E10.5, 11.5, and 12.5 in the whole brain), pre-microglia [E14 and postnatal days (P)3, 6, and 9 in the whole brain] and adult microglia (8 weeks old in the cortex, hippocampus, and spinal cord). These clusters displayed similarities within themselves across time points but were different from each other (47). In this study, early microglia–associated genes included those involved in cell cycling and differentiation (e.g., *DAB2* and *MCM5*), whereas genes associated with neuronal development (e.g., *CSF1* and *CXCR2*) were found in the pre-microglia cluster. Canonical adult microglial genes (e.g., *CD14* and *PMEPA1*) were also primarily expressed in adult microglia (47). When investigating the chromatin landscape across these time points, a similarity between early microglia and pre-microglia was revealed, perhaps indicating that alterations in chromatin accessibility occur prior to bulk RNA changes (47); specifically, chromatin alterations were associated with changes in the accessibility of enhancers, but not promoters (47). With single-cell RNA sequencing (scRNA-seq), the authors also discovered that *MCM5*, *CSF1*, and *MAFB* denote early, pre-, and adult microglia, respectively (47). Furthermore, analysis of the expression of TFs indicated that some of them appear in pre-microglia and continue to be expressed in adult microglia (*EGR1* and *SALL1*), whereas some TFs were specific only to adult microglia (*FOS*, *JUN*, *MAFB*, and *MEF2A*) (47).

Another study in mice revealed differences in CD45^{int}CD11b^{+} microglial clusters between E16.5 (in the cerebellum, spinal cord, forebrain and midbrain) and postnatal weeks 3 (juvenile) and 16 (adult) (in the cerebellum, spinal cord, cortex, hippocampus, corpus callosum, and facial nucleus) (46). In all examined ages, the proportion of different microglial clusters varied across regions, with the adults showing the most homogeneity between regions (46). Specifically, in the juvenile animals, there was a considerable similarity between genes expressed in the cortex, hippocampus, and forebrain versus the cerebellum and spinal cord, which presented a great degree of overlap (46). Temporally, embryonic microglia were also differentiated from postnatal microglia by their low expression of *MALAT1*, *SELPLG*, *SLC2A5*, and *TMEM119*, as well as their expression of *APOE*, *CTSB*, and *TMSB4X* (46). In embryonic microglia, 6 clusters were differentiated by *CTSB*, *CTSD*, *EEF1A1*, *LAMP1*, and *RPL4*, potentially indicating that some clusters had increased

lysosomal activity (46). Between the juvenile and adult cohorts, CST3 and SPARC varied temporally and regionally. In the cortex, most microglia were double-positive for CST3 and SPARC proteins in juveniles, and while this was still the case in adulthood, it was in a smaller percentage of cells, with the emergence of $CST3^{neg}SPARC^{+}$ cells. In the cerebellum, the percentage of microglia being $CST3^{+}SPARC^{neg}$ and $CST3^{+}SPARC^{+}$ was, by contrast, consistent over time (46). CST3 is a widely expressed protease inhibitor that was found to be upregulated in patients with multiple sclerosis (MS) (31). Intriguingly, recent work showed that administration of CST3 exacerbated experimental autoimmune encephalitis (EAE) (a model of MS) in 8- to 12-week-old female, but not male, mice, which was accompanied by more (peripheral) immune cells in the spinal cord (31). There were also increased levels of antigen presentation costimulatory molecules (i.e., CD80 and CD86) in $CD11b^{+}$, but not $CD11c^{+}$, cells and therefore likely increased levels in monocytes and macrophages but not dendritic cells (31). Hence, it is possible that increased CST3 in the juvenile time period could be serving an immunogenic role in some microglia, perhaps increasing their phagocytic activity at a time when extensive neural refinement and plasticity are underway (46). SPARC is highly expressed in astrocytes and microglia during development, with its levels decreasing in adulthood; however, its levels also increase in the contexts of injury and neurodegeneration (33, 65). Intriguingly, SPARC has been linked to increased glutamate receptors and altered neuronal plasticity (33); therefore, it will be relevant to determine why SPARC remains upregulated in microglia from the cortex into adulthood during normal physiological conditions, outside of injury or disease.

A recent preprint has analyzed $CD11b^{+}CD45^{low}CD64^{+}CX3CR1^{high}$ cortical microglia isolated from human fetuses during early to mid-gestation (gestational weeks 9–17) and from epileptic resections of pediatric and adult patients (27). Fetal microglia, compared to postnatal microglia, displayed a greater expression of *ACTB*, *FTL*, and *SPP1*, as well as of transcripts related to cell proliferation (*CDK1*, *CDK5*, *HMGA1*, *PTMS*, and *STMN1*) and oxidative phosphorylation (*ATP1B1*, *ATP6V1F*, *COX5A*, *COX8A*, *NDUFA3*, and *NDUFA13*), and had a lower expression of transcripts related to immune function (*CD4*, *CD74*, *HLA-B*, *HLA-DRA*, *IL1B*, *IL4*, *ITGAM*, and *TNF*) (27). When compared to the rest of the cortical tissue, microglia at all time points had common upregulated transcripts (*C1QA*, *C3*, *CSF1R*, *CX3CR1*, *IL1B*, *IRF5*, *IRF8*, *P2RY12*, *TGFBR2*, *TMEM119*, and *TNF*), whereas some transcripts selectively defined microglia in the fetal cortices (*APOBEC3B*, *APOE*, *BIN1*, *CSF1*, *CTSD*, *CRYBB1*, *IGF1*, *IL7R*, *FTL*, *MEF2C*, *MITF*, and *SALL1*) and postnatal cortices (*FKBP5*, *IGF2BP3*, *HSPA1B*, *LA-DQB1*, *MALAT1*, *MX2*, *RUNX2*, and *SGK1*) (27). Using a program that predicts ligand–receptor interactions, Han et al. (27) further showed that *DLL1* and *IGF1* predicted the fetal microglial transcriptome, whereas *BMP7*, *TGFB1*, and *TGFB3* predicted the postnatal microglial transcriptome. In addition, assay for transposase-accessible chromatin with sequencing (ATAC-seq) performed in a number of embryonic and postnatal samples was used to assess TF activity, revealing that CEBPA, CEBPD, and MAF were highly active in the fetal samples, and AP-1 and SMAD3 in the postnatal samples (27).

Together, these studies reveal that microglia exhibit temporally regulated transcriptomes and point toward potential functions (such as cell differentiation, proliferation, and phagocytosis) specifically exerted across critical periods of development. In addition to the analysis outlined above, recent work has also defined certain transcriptionally defined microglial states, which are present during development and described below.

2.1.1. Proliferation-associated microglia. In one study, microglia from mouse brains (from the olfactory bulb, cortex, striatum, hippocampus, and cerebellum) displayed heterogeneity, with multiple clusters appearing at P7 (38). By contrast, microglia examined in adulthood were found to be more homogeneous (38). From these clusters observed at P7, a $GPNMB^{+}SPP1^{+}$ cluster appeared

in white matter regions, including the corpus callosum and white matter areas of the cerebellum, which are both considered gliogenic and neurogenic areas (in other words, areas where cellular proliferation is abundant); therefore, Li et al. (38) termed these cells proliferation-associated microglia (PAM). PAM had a somewhat downregulated expression of genes associated with microglial homeostatic functions (*P2RY12*, *SALL1*, *SIGLECH*, *TGFBR1*, and *TMEM119*) and an upregulated expression of genes found in other defined transcriptional signatures—for example, disease-associated microglia (DAM) (35, 37) (see Section 2.3.1)—reported under pathological conditions (*APOE*, *CD9*, *CD63*, *CLEC7A*, *FABP5*, *GPNMB*, *IGF1*, *ITGAX*, *LGALS3*, *LPL*, *SPP1*, and *TYROBP*) (38). Furthermore, these PAM, more than other subsets of microglia or other myeloid cells, such as macrophages, monocytes, and natural killer (NK) cells, had an upregulated expression of *SPP1* and *GPNMB* (38). Additionally, the appearance of PAM, unlike some other defined transcriptional signatures (e.g., DAM, described in Section 2.3.1) (35, 37), was not dependent on TREM2 or APOE (38). The authors showed that CLEC7A$^+$ microglia (presumed PAM) observed in situ in these regions had increased phagocytic capacity and contained pyknotic nuclei (associated with apoptosis), indicating their likely phagocytosis of newly formed, but dying, oligodendrocytes (and perhaps, although to a much lesser degree, astrocytes) (38), thus suggesting that SPP1 and GPNMB may be involved in microglial regulation of axonal myelination.

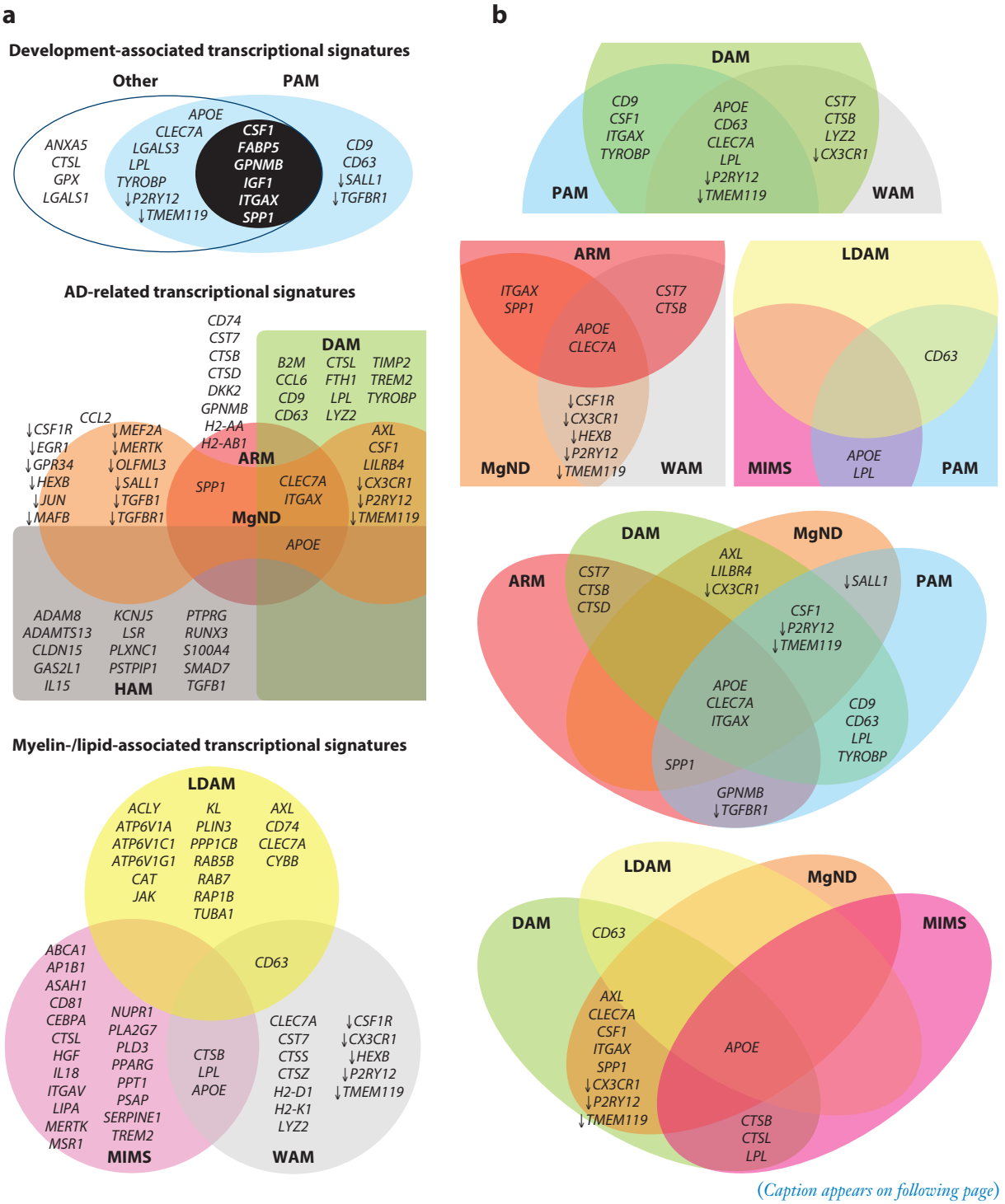
Intriguingly, as an example, *SPP1* expression was also found to be upregulated in the spinal cord during aging, and it was associated with increased demyelination and axonal loss in the spinal cord of adult female mice following oxidized phosphatidylcholine injury (16, 79). Dong et al. (16) showed that local administration of SPP1 increased demyelination and axonal loss, while the knocking-down of SPP1 attenuated pathology; additionally, SPP1 increased microglial expression of proinflammatory factors (16). This study presents the possibility that SPP1 drives microglial phagocytosis to clear myelin or myelin-producing cells at a time when it is required—during development or in the acute injury response. Future work is required to identify what regulates SPP1 in microglia because in the adult brain its expression is not elevated outside of disease states, yet it is elevated during development. Furthermore, many cell types can express SPP1, so any potential therapeutic targeting must consider its expression pattern.

Conversely, GPNMB is an endogenous glycoprotein whose role is still unclear, although reports indicate that it may have an immunomodulatory or immunoregulatory function (51). GPNMB is regulated by progranulin, a secreted glycoprotein that is implicated in a number of neurodegenerative diseases (32). A recent study showed an interesting sex-specific role for GPNMB (32). In adult progranulin knockout mice, females displayed increased GPNMB protein levels in microglia, whereas males had reduced levels of GPNMB in myeloid cells and microglia (in the whole brain) (32); this was associated with a proinflammatory and neurotoxic environment in the male, but not the female, progranulin knockout mice (32). Therefore, it may be that during development, GPNMB is upregulated in PAM to help limit microglial reactivity, in other words, contributing to normative phagocytosis (perhaps associated with SPP1). Then, in disease contexts, such as MS or AD, which have sex differences in their prevalence and progression (15), GPNMB functioning may become altered in a way that promotes microglial reactivity or an enhanced proinflammatory state, leading to reduced tissue repair. Or perhaps, as GPNMB appears to be involved in lipid regulation, it could be upregulated during peak lipid clearance in postnatal development. Microglial lipid processing capacity is reduced throughout the life span (43), which may be due to reduced GPNMB expression; therefore, understanding its regulation into adulthood could be potentially impactful for therapeutics. More work is also needed to understand any potential functional and regulatory relationship between SPP1 and GPNMB, particularly in microglia, as these genes are often found to be upregulated together in microglia during disease conditions/immune challenges in humans and rodents (34).

2.1.2. Axon tract–associated microglia and unnamed signatures in development reminiscent of proliferation- and disease-associated microglia. Other studies have examined microglia in development and found transcriptional signatures related to PAM and DAM. An RNA-seq study in mice revealed that neonatal (P4–7) CD11c^{+} microglia upregulate a number of transcripts when compared to CD11c^{neg} microglia (*ADAM19*, *BMP2*, *CSF1*, *CXCL12*, *GPNMB*, *GPX3*, *IGF1*, *LGALS1*, *MMP12*, *MMP19*, *MMP24*, *NTN1*, *PLXNA2*, *PTN*, *SEMA7A*, and *SPP1*) (78). These elevated transcripts represent genes involved in the promotion of neuronal, astrocyte, and oligodendrocyte support and differentiation, among other key functions. In addition, in CD11c^{+} microglia, IGF1 was necessary for proper developmental myelination (78). Another study examining microglia (CD11b^{+}CD45^{+} cells) in mice revealed that corpus callosum microglia, compared to cortical microglia, at P7, had increased expression of similar genes (*ATP6V0D2*, *CLEC7A*, *GPNMB*, *IGF1*, *ITGAX*, and *SPP1*), as assayed by microarray (25). Cells with a similar signature were not limited to the white matter, although they were often associated with it. For example, another study demonstrated a gene expression signature similar to that of PAM/DAM when examining bulk-sequenced microglia (CX3CR1^{+}CD45^{+}CCR2^{neg} cells) isolated from the mouse retina at P7, but not E15.5 or P60 (*ABHD4*, *ANK*, *APOE*, *AXL*, *CD36*, *CD68*, *CLEC7A*, *CSF1*, *CTS7*, *CTSB*, *CTSL*, CTSZ, *GAS7*, *IGF1*, *ITGAX*, *LAMP1*, *LGALS3*, *LPL*, *LYZ2*, *NRAP*, *SLC6A6*, *SOD1*, *SPP1*, and *TYROBP*) (3). Intriguingly, in the CD11c^{high} cells within these microglia (CX3CR1^{+}CD45^{+}CCR2^{neg} cells), there was increased expression of *CLEC7A*, *IGF1*, *ITGAX*, and *LPL* and downregulation of homeostatic markers, such as *P2RY12* and *TMEM119* (3). Cells with this transcriptional signature were associated with apoptotic neurons, as well as TREM2 and APOE signaling; however, this signature was not associated with CSF1R, as these cells were resistant to CSF1R pharmacological inhibition or knockdown-induced depletion (3). A follow-up study by the same group using scRNA-seq further showed the occurrence of 11 different clusters, many of which resembled clusters/signatures observed in other studies (2).

Another study provided scRNA-seq of microglia from the whole mouse brain at a number of time points, including E14.5; P4–5; and 1, 3, and 18 months of age, as well as from the white matter of adult mice subjected to lysolecithin injury causing demyelination (26). From these data, a cluster of microglia (*ANXA5*, *CTSL*, *IGF1*, *FABP5*, *GPNMB*, *LGALS1*, *LGALS3*, *LPL*, and *SPP1*) emerged at P4–5. Putative cells from this cluster (SPP1^{+}) were predominantly located in the corpus callosum and white matter tracts of the cerebellum, and were termed axon tract–associated microglia (ATM) (26). Intriguingly, not all microglia in these white matter regions were SPP1^{+}, which implies, potentially, that these ATM cells may be somehow differently programmed regardless of influence from the regional milieu (26).

These similarities have led some to consider that perhaps microglia with similar transcriptional signatures identified across these studies (PAM, ATM, and others listed above) (2, 3, 25, 26, 38, 78) represent the same microglial state (7). This is bolstered by an overlap between the expressed transcripts (**Figure 1*a***). A number of transcripts were expressed in microglia across all (*CSF1*, *FABP5*, *GPNMB*, *IGF1*, *ITGAX*, and *SPP1*) or most (*ANXA2*, *ANXA5*, *APLP2*, *ATF3*, *ATP1A3*, *ATP6V0D2*, *BNIP3*, *CD28*, *CCL3*, *CCL9*, *CLEC7A*, *COLEC12*, *CRIP1*, *EPHX1*, *FAM20C*, *FORL2*, *GM1673*, *GPX3*, *HPSE*, *IFITM2*, *LAG3*, *LGALS1*, *LILRB4*, *LPL*, *NCEH1*, *PLAUR*, *PLD3*, *PLIN2*, *S100A1*, *SLC16A3*, *SPP1*, and *VAT1*) studies (7). Intriguingly, some of the transcripts, such as *VAT1*, were also associated with other cell types (e.g., cholinergic synaptic vesicles), which could be indicative of messenger RNA from phagocytosed cells. Furthermore, these clusters often downregulated more homeostatic-associated genes such as *P2RY12* and *TMEM119* (7). It has been postulated that this could be due to the use of different methods for cell isolation and transcriptomic techniques, which is a tempting hypothesis (7). While the resemblances and differences between these cells remain to be unraveled, it would also be important to focus on identifying



(*Caption appears on following page*)

Figure 1 (*Figure appears on preceding page*)

Similarities and differences of highlighted transcripts across different microglial states. Transcripts listed are not exhaustive but rather those highlighted across the various originating studies. (*a*) Three cases of highlighted microglial transcriptional signatures across semi-similar contexts: development-associated, AD-related, and myelin-/lipid-associated. In the development-associated transcriptional signatures, the inner black circle represents transcripts altered similarly across all studies (2, 3, 7, 25, 26, 38, 78), whereas the light-blue oval (*right*) includes those upregulated in PAM and the dark blue–outlined oval (*left*) includes those upregulated in the majority (but not all) of the other described signatures. AD-related transcriptional signatures include DAM (*green vertical rectangle*, *right*) (35), MgND (*orange dumbbell*, *middle*) (37), ARM (*red circle*, *center*) (53) and HAM (*gray horizontal rectangle*, *bottom*) (61). Only APOE is expressed in all four signatures and is the only transcript from the highlighted HAM transcriptional signature to overlap with DAM, MgND, or ARM. Myelin-/lipid-associated transcriptional signatures include LDAM (*yellow circle*) (43), MIMS-foamy (*pink circle*) (1), and WAM (*gray circle*) (52). LDAM, MIMS-foamy, and/or WAM share little overlap of highlighted transcripts. (*b*) Overlap of highlighted transcripts from different microglial transcriptional signatures. PAM and/or WAM have a number of transcripts overlapping with DAM, MgND, and/or ARM, whereas there are fewer overlapping transcripts between PAM, WAM, DAM, MgND, and/or ARM with LDAM and/or MIMS. The only highlighted transcript that overlaps between LDAM and other microglial transcriptional signatures is CD63. APOE is altered across most microglial transcriptional signatures, upregulated in all except LDAM. As all transcripts listed are not exhaustive, but rather represent those highlighted across the various originating studies, this does not preclude overlap of other transcripts besides those listed or overlap of transcripts listed that are not captured by these diagrams. Subsequent analysis should be performed on full lists of altered transcripts across putative microglial states and transcriptional signatures to understand the similarities and differences beyond the emphasized transcripts. Abbreviations: AD, Alzheimer's disease; ARM, activated response microglia; DAM, disease-associated microglia; HAM, human Alzheimer's disease microglia; LDAM, lipid droplet–accumulating microglia; MgND, microglial neurodegenerative phenotype; MIMS, microglia inflamed in multiple sclerosis; PAM, proliferation-associated microglia; WAM, white matter–associated microglia. Figure created with modified shapes from VennPainter (40).

the specific functions of the associated microglial transcripts at these pivotal developmental timepoints and among regions where they are enriched (e.g., the corpus callosum and white matter tracts of the cerebellum and retina).

2.2. Adult Steady-State/Homeostatic Functioning

An early study in the field conducted in adult mice showed that microglia ($CD11b^+CD45^{low}$ cells) had an enriched expression of a number of transcripts (*C1QA*, *CD34*, *FCRLS*, *GPR34*, *HEXB*, *MERTK*, *OLFML3*, *P2RY12*, *PROS1*, *SALL1*, *TGFBR1*, and *TMEM119*) compared to $CD11b^+Ly6C^+$ monocytes (11). The authors also found these transcripts to be expressed in microglia from human fetal and post-epilepsy surgical resection samples (temporal lobe), with *C1QA*, *GPR34*, *MERTK*, *P2RY12*, and *PROS1* also being upregulated or uniquely expressed in microglia ($CD11b^+CD45^{low}$ cells) (11).

Furthermore, in mice where TGFB1 is only expressed in T cells, $CD11b^+CD45^{low}P2RY12^+$ microglia were absent from the brain, even though a smaller number of $IBA1^+$ cells remained (11). This signature appears in mice during the early postnatal period, even though other signatures coexist, and is maintained until adulthood (11). Other studies have revealed a similar microglial transcriptional signature in adult mice and humans (8, 26, 29, 38, 46, 54, 55). Therefore, the most common (in terms of both being studied and enrichment/expression in only microglia) transcripts and markers that are attributed to this state (often termed typical, homeostatic, or normal adult microglia in literature) are CSF1R, CX3CR1, HEXB, P2RY12, and TMEM119.

2.2.1. Regional differences. There are potential differences in microglial signatures across regions. An initial study in $CD11b^+$ cells (enriched for microglia, but not limited to these cells) from 3-month-old male mice showed regionally varied transcription profiles between the cerebellum, cortex, striatum, and hippocampus, with the cerebellum being particularly different (21). Microglia in the hippocampus and cerebellum specifically presented an upregulation of genes that were associated with immune processes, including pathogen recognition (*CLEC7A*), interferon pathway genes (*IFIT2*, *IFITM3*, *IRF7*, *OASL1*, *PLSCR1*, *STAT1*, and *STAT4*), and antigen

presentation (*CD74*, *H2-AA*, *H2-AB1*, *H2-D1*, *H2-EB1*, and *H2-K1*), as well as those associated with energy metabolism, including generation of precursor metabolites and energy (glycolytic pathway), the electron transport chain, oxidative phosphorylation processes (*CAT*, *GPX4*, *GPX8*, *PRDX2*, *PRDX5*, *SOD1*, and *SOD2*), and overall energy regulators (*PPARG*) (21). This led the authors to conclude that microglia from the hippocampus and cerebellum, compared to the other regions examined, might exist in a state that is more ready to respond to immune challenges, which was corroborated by their patterns of gene expression (*CD47*, *CD200R4*, *CD300A*, *CD300LB*, *CD300LD*, *CD300LF*, *SIGLEC2*, *SIGLEC3*, *SIGLECE*, *SIGLECH*, *SIRPA*, *SIRPB1A*, *TREM1*, *TREM2*, and *TREM3*) (21). The same study revealed that cerebellar microglia remained distinct from those in other regions over the course of aging, between 4, 12, and 22 months (21). By contrast, hippocampal microglia, which were distinct from those in all other regions during young adulthood at 4 months, were more similar to microglia in the striatum and cortex in middle age, at 12 months, but still remained distinct—a difference that disappeared in aging at 22 months (21). Furthermore, throughout aging, there was a global downregulation of microglial signature homeostatic genes (*FCRLS*, *P2RY12*, *P2RY13*, and *TMEM119*) in all examined regions, with the greatest decrease occurring in the cerebellum (21).

However, work using deep scRNA-seq and bulk sequencing on $CD45^{low}CD11b^{+}$ cells from young adult mice found very few differences between regions (cortex, cerebellum, hippocampus, and striatum) in the typical adult microglial cluster (38). Furthermore, the authors attributed transcripts that contributed to the regional differences mentioned above (i.e., *CD177*, *CD290*, *CLEC4A*, *FPR1*, *H2-AA*, *H2-EB1*, *KLRA2*, *SLAMF7*, and *TREM3*) (21) to being primarily found in clusters that were not microglia but other monocyte populations (38). Yet, there was also expression of some of these transcripts (*H2-AA*, *H2-EB1*, and *TREM3*) in their typical adult microglial cluster (38).

Additional research has shown differences between regions, particularly when analyzing cells sorted using more specific microglial markers. For example, bulk sequencing of $TMEM119^{+}$ cells from 2-month-old mice exhibited differences between the cortex, striatum, and midbrain (5), although this analysis could benefit from an unbiased approach to investigating differences between all three brain regions. In whole-mouse cortices and spinal cords from 2-month-old mixed sex samples, scRNA-seq also revealed microglial clusters (enriched in *CX3CR1*, *P2RY12*, and *TMEM119*) that were different between these regions (81). In the cortex, there were two clusters associated with homeostatic function [with a downregulation of ribosomal genes (*RPS11*, *RPS21*, and *RPL26*)] and one smaller cluster of cells in which immune system–associated genes were upregulated (*CCL3*, *CCL4*, *IL1A*, and *IL1B*), which were termed inflammatory microglia by the authors (81). In the spinal cord, however, there were two approximately similar-sized clusters, one of homeostatic microglia and one of inflammatory microglia (81). This showed potential regional differences, perhaps not of the clusters or transcripts altered, but of their proportions, which largely held consistent at 4 and 8 months of age in both the cortex and spinal cord (81). As the spinal cord was not considered in other studies examining regional differences of microglial transcriptomics, whether microglia located in this region exhibit varied transcriptomes compared to the cortex (and potentially other CNS regions) remains to be determined.

It is clear that there are regional differences in microglial transcriptomes throughout development, and even subregional differences (e.g., cerebellum white matter) (26, 46, 78). However, the question of microglial transcriptional regional heterogeneity in rodents, particularly in the adult steady state, is far from being settled. Furthermore, these regional analyses are limited in their scope, generally focusing on the same larger regions, such as the cortex and striatum, whereas many subcortical, mid-, and hind-brain regions have not yet been given robust regional comparisons. The use of in situ methodologies could allow for the analysis of regions not typically

suitable for common sorting workflows, whether because of an inability to dissect or a limited number of viable cells likely to be obtained (potentially necessitating an increased use of animals). Care needs to be taken, of course, in highlighting whether the assayed cells are microglia, as it should also be noted that some microglial signatures do have downregulated levels of some more homeostatic markers (e.g., *P2YR12* and *TMEM119*). Therefore, it is important to clarify [as was done in the study mentioned above (38)] that in cases where no regional differences were found, this is perhaps reflective of only the typical adult microglia, which dominate microglial numbers at this time point and may obscure regional differences in other less abundant signatures that may still be having a functional impact.

Studies in human microglia also revealed some regional differences. A study in which microglia (a subset of $CD45^+$ cells) were isolated from tissue absent of pathology from patients undergoing surgical resection for epilepsy, glioma, or metastasis showed differences along a spatiotemporal axis (54). In most microglial clusters identified in the study, there were differences in their prevalence between white and gray matter (54). Furthermore, microglia in gray matter had increased expression of *APOE*, *CD68*, *CX3CR1*, *EMR1*, and *HLA-DR* compared to those in white matter (54). Further cluster analysis revealed differences in most microglial clusters across age (<30 years old versus 30 to 50 years old versus >50 years old) (54). Finally, in microglia ($IBA1^+$ cells) from white matter, $SPP1^+$ microglia varied along a temporal axis, with increasing age, to reach a final average of 10% of microglia (54). In gray matter, $SPP1^+$ microglia also increased along a temporal axis, but only as many as 2.5% of microglia were $SPP1^+$ (54). SPP1 was also highlighted in developmental signatures associated with white matter (described above) and in the spinal cord of aged mice (16, 79). Future work should continue to investigate the functional relevance of this protein expressed in microglia across these many contexts. Additionally, future work utilizing human samples should as much as possible investigate regional differences, noting that surgical resection samples are often isolated from somewhat similar regions (e.g., the temporal lobe).

2.2.2. Sex differences. The assessment of sex differences by analyzing transcriptomics is less robust, although many rodent studies utilize mixed samples of both sexes, while human studies include samples across sexes. Research along a temporal axis showed that there are remarkably few sex differences in microglial transcriptional signatures across multiple time points, including at E14.5, P4–5, and 1, 3, and 18 months in microglia ($CD45^{low}CD11b^{high}CX3CR1^{high}$) from whole-brain samples (26). However, at 13 weeks, microglia (a subset of $CD11b^+$ cells) from male and female mice exhibited different transcriptional signatures in the hippocampus and cortex (24), which may indicate a sex-by-region effect driving these differences. On the other hand, in a large cohort study of microglia (a subset of $CD11b^+$ cells) isolated from humans, sex was the one factor that did not significantly contribute to microglial variation, in contrast to disease, age, brain region, dominant hemisphere, and ancestry, which all significantly contributed (80). Yet, there were a number of transcripts that differed between men and women (80). Therefore, despite the many documented sex differences in microglial structure and function (10, 15, 41), how sex contributes to modulating their transcriptionally defined signatures requires further exploration.

2.3. Microglia in Disease Conditions

There are numerous microglial signatures that have been identified transcriptionally across a wide range of pathologies, in both rodents and humans (**Figure 1**).

2.3.1. Disease-associated microglia. In 6-month-old 5xFAD mice (a model of AD pathology), analysis of brain $CD45^+$ cells that underwent massively parallel RNA single-cell sequencing (MARS-seq) revealed three clusters of microglia, of which a single cluster was readily expressed

in age-matched wild-type animals, with the cells in the remaining two clusters termed DAM (35). These cells downregulated genes associated with homeostatic microglia (*CX3CR1*, *P2RY12*, and *TMEM119*) and upregulated other genes (*CD9*, *CD63*, *CLEC7A*, and *ITGAX*), including those associated with AD risk factors (*APOE*, *CTSD*, *LPL*, *TREM2*, and *TYROBP*) (35). Of the two DAM clusters, one had greater expression of these signature associated markers, which the authors took to indicate that the cluster with lesser expression was an intermediary one (35). Interestingly, the putative intermediate signature was not dependent on TREM2, which was necessary for the development of DAM (35). The intermediary signature was associated with a decrease of homeostatic genes (*CX3CR1*, *P2RY12*, and *TMEM119*) and an increase in other genes associated with the signature (*APOE*, *B2M*, *CTSB*, *CTSD*, *FTH1*, *LYZ2*, and *TYROBP*), while DAM were associated with further upregulation of DAM genes (*AXL*, *CCL6*, *CD9*, *CLEC7A*, *CSF1*, *CST7*, *CTSL*, *ITGAX*, *LILBR4*, *LPL*, *TIMP2*, and *TREM2*) (35). During postnatal development into adulthood (1, 3, 6, and 8 months), the proportion of $CD11c^+$ microglia that were considered DAM increased, exhibiting a plateau at 3 and 6 months, then progressed greatly at 8 months (35). Putative DAM in situ, which were enriched for CD11c, were apposed to plaques in the AD pathology mouse model; while in humans, LPL^+ microglia, also considered as putative DAM, were adjacent to plaques in postmortem samples from individuals with AD and contained thioflavin-S-labeled particles (which may indicate phagocytosis of plaque material) (35). These cells were also present in the spinal cord of a mouse model of amyotrophic lateral sclerosis (ALS) pathology and showed increased prevalence with disease progression (35).

2.3.2. Microglial neurodegenerative phenotype. In another study (37), microglial ($FCRL^+$) transcriptional profiling was assayed in mice: an APP-PS1 model of AD pathology over the course of aging (2, 7, 10, and 17 months), an EAE model of MS at different disease stages, and an ALS pathology SOD1 model across disease stages (37). Among these conditions, there were two gene clusters that emerged: one with a downregulation of homeostatic genes (*CSF1R*, *CX3CR1*, *GPR34*, *HEXB*, *JUN*, *MERTK*, *OLFML3*, *P2RY12*, *RHOB*, *TGFB1*, *TGFBR1*, and *TMEM119*) and TFs (*EGR1*, *JUN*, *MAFB*, *MEF2A*, and *SALL1*) and one with an upregulation of genes associated with immune-related genes (*APOE*, *AXL*, *CCL2*, *CLEC7A*, *CSF1*, *ITGAX*, *LILRB4*, and *SPP1*) termed microglial neurodegenerative phenotype (MgND) (37). Across the three disease models, there was a negative correlation of microglial *MEF2A*, *SALL1*, and *TGFBR1* and a positive correlation of *APOE* with disease progression (37). Furthermore, with EAE, there was cycling of suppression and restoration of microglial homeostatic genes (*EGR1*, *MAFB*, *MEF2A*, *P2RY12*, *SALL1*, *TGFBR1*, and *TMEM119*) as the disease model went between the acute phase and recovery (37). Similarly, MgND microglia did not suppress T-cell proliferation in the spinal cord of EAE mice, contrary to homeostatic microglia from nondiseased spinal cords (37). In the APP-PS1 model, CLEC7A, as a marker of MgND, was associated in situ with plaques, and there was a transcriptional overlap between $CLEC7A^+$ microglia and MgND (37). The authors highlighted that APOE and TGFB are both major upstream regulators of MgND (37). Additionally, APOE regulated the transcriptional profile of MgND, but only in those actively phagocytosing; furthermore, TREM2 regulated this APOE pathway (37). In human postmortem samples from patients with AD, mutations of TREM2 downregulating its function also led to less abundant microglia surrounding plaques (37).

2.3.3. Activated response microglia. This signature of microglia was profiled in APP(NL-G-F) mice, an APP knock-in model of AD pathology, which was assayed over multiple time points corresponding to different phases of disease pathology (3, 6, 12, and 21 months) (53). Analysis of $CD11b^+$ cells from the cortex and hippocampus revealed six clusters of microglia, two of which were associated with homeostatic microglia, while the other four were considered reactive (53). Activated response microglia (ARM), representing one of these reactive clusters,

showed an upregulated expression of *APOE* and of genes related to inflammatory processes (*CLEC7A*, *CST7*, and *ITGAX*), major histocompatibility complex (MHC) presentation (*CTSB*, *CTSD*, *CD74*, *H2-AA*, and *H2-AB1*), and tissue regeneration (*DKK2*, *GPNMB*, and *SPP1*) (53). A related cluster, transiting response microglia, had a similar profile but with a lower expression of these genes (53). An interferon response microglial cluster also displayed increased expression of interferon response type 1 pathways (*IFIT2*, *IFIT3*, *IFITM3*, *IRF7*, and *OASL2*) (53). These three microglial clusters increased throughout disease progression, with homeostatic microglia potentially transitioning along either the ARM pathway or interferon response microglia pathway (53). The final cluster, cycling/proliferating microglia, represented a small subset of all microglia and expressed genes involved in DNA replication, chromatin rearrangement, and cell cycle (*CDK1*, *MCM2*, *MKI67*, *TOP2A*, and *TUBB5*) (53). In situ, $APOE^+$ microglia, representing putative ARM, were also observed to cluster around plaques, which was blunted by APOE knockdown (53). Furthermore, knockdown of APOE reduced the proportion of ARM but actually increased that of the interferon response microglial cluster in the examined AD pathology model (53).

2.3.4. Human Alzheimer's disease microglia. This subset was identified using frozen frontal cortices (superior frontal gyri) from postmortem human samples with AD (61). Microglia (a subset of $CD11b^+$ cells) showed increased expression of *ABCA7*, *APOE*, *GPR141*, *PTK2B*, *SPI1*, and *ZYX* and downregulated expression of *MEF2C* (61) versus other cells. The differential expression with AD included increases of the following transcripts: *A1BG*, *ACD*, *ADAM8*, *ADAMTS13*, *APOE*, *ARSA*, *ATOH8*, *CBX6*, *CHCHD5*, *CLDN15*, *CTBP1-AS*, *DPYD*, *EMP2*, *FAM109A*, *FBRSL1*, *FOXP1*, *GAS2L1*, *GYPC*, *IL15*, *LOC100133445*, *LOC100507639*, *LOC102724549*, *LOC102725328*, *LSR*, *KCNJ5*, *PLXNC1*, *PSTPIP1*, *PTPRG*, *RFX2*, *RUNX3*, *SECTM1*, *S100A4*, *SLC38A7*, *SMAD7*, *SMIM3*, *STEAP3*, *TGFB1*, *TM9SF1*, *TSHZ3*, *TTYH3*, *ULK*, *VENTX*, *ZNF696*, *ZNF703*, and *ZNF843*. It included decreases in *ANKRD26P3*, *ASTN1*, *CECR2*, *GLT1D1*, *GRIA2*, *HIST2H2BA*, *IGSF10*, *LOC102724661*, *MEIS1*, *MOV10L1*, *NIN*, *PDCD6IPP2*, *PTPRZ1*, *RIMS2*, *SELENBP1*, *SERPINF1*, *TLN2*, *TNFRSF21*, *ZBTB8B*, *ZNF532*, and *ZNF662*. These results indicate a differential expression signature defining HAM (61). These cells were largely unrelated to clusters observed in mouse studies, such as DAM, MgND, or ARM (61), although they similarly increased APOE (**Figure 1*a***; see also the sidebar titled DAM, MGND, ARM, and HAM: How Similar?).

2.3.5. Glioma-associated microglia. Through analyzing multiple murine RNA-seq data sets, some genes were found to be differentially expressed only in glioma samples and not in ALS

DAM, MGND, ARM, AND HAM: HOW SIMILAR?

Analysis of the data from multiple studies indicates that disease-associated microglia (DAM) (35), the microglial neurodegenerative phenotype (MgND) (37), and activated response microglia (ARM) (53) may represent a similar state with slight contextual variations. Together, the data may suggest that it is possible that in many pathological conditions, when there is phagocytosis (particularly of apoptotic cells, such as in neurodegeneration), there is potentially a common transitory microglial state induced in some stepwise manner, with APOE and TREM2 signaling being recruited. It is unclear, however, whether a similar microglial state occurs in humans. Human Alzheimer's disease (AD) microglia (HAM) show very little overlap with DAM, MgND, and ARM, although they all upregulate APOE (61). At least one study highlighted in humans the overlap of microglial transcripts upregulated across diseases [e.g., multiple sclerosis (MS), AD, and amyotrophic lateral sclerosis (ALS)], in particular APOE and SPP1, which are upregulated in DAM, MgND, and ARM (39).

and AD pathologies (13). These included an upregulation of *BAIAP2*, *CXCL11*, *GBP4*, *IFI47*, *IKBKE*, *ITGAL*, *LRRC8C*, *OAS1G*, *OAS2*, *RNF213*, and *STAT1* and a downregulation of *ERRFI1*, *SCUBE1*, *STK26*, *SRL*, and *ZFP212* (13). Using these genes as targets, the authors showed that ITGAL (CD11a) was upregulated in pilocytic astrocytoma, a type of glioma (13). Furthermore, targeting CD11a with a neutralizing antibody reduced glioma proliferation in a mouse model (13).

2.3.6. Microglia inflamed in multiple sclerosis. In this study, snRNA-seq was performed on frozen human postmortem samples from white matter MS lesions across stages of inflammation, the demyelination lesion core, and white matter periplaque (1). Multiple microglia inflamed in multiple sclerosis (MIMS) clusters were exposed, including foamy and iron (1). The foamy cluster showed increases in transcripts associated with lipid storage (*ABCA1*, *IL18*, *ITGAV*, *LPL*, *MERTK*, *MSR1*, and *PPARG*), lipoproteins (*ABCA1*, *APOE*, *CD81*, *LPL*, and *PPARG*), lysosomal activity (*AP1B1*, *ASAH1*, *CTSB*, *CTSL*, *LIPA*, *PPT1*, and *PSAP*), and inflammatory response regulation (*APOE*, *CD81*, *CEBPA*, *HGF*, *IL18*, *LPL*, *NUPR1*, *PLA2G7*, *PLD3*, *PPARG*, *SERPINE1*, and *TREM2*), in addition to presenting a foamy morphology in situ (1). The iron cluster had increases in transcripts associated with ribosomal proteins, MHC class II (*CD74*, *HLA-DRA*, and *HLA-DPA1*), ferritin (*FTH1* and *FTL*), Fc receptors (*FCGR2A*, *FCGR3A*, and *FCGRT*) and the complement system (*C1QA* and *C1QB*), with the complement system being potentially important in determining this cluster (1).

2.3.7. Lipid droplet–accumulating microglia. Transcriptional analysis was performed in $CD11b^{+}CD45^{low}$ microglia from the hippocampus of 18-month-old mice (43), separating lipid droplet low and high signatures. Genes enriched in the lipid droplet high signature included those related to lysosomal activity (*ATP6V1A*, *ATP6V1C1*, *ATP6V1G1*, *CD63*, and *TUBA1*), vesicular transport (*RAB5B* and *RAB7*), nitric oxide and reactive oxygen species (ROS) generation (*CAT*, *JAK*, *KL*, *PPP1CB*, and *RAP1B*), and lipid-related genes (*ACLY* and *PLIN3*), as well as a downregulation of homeostatic genes (*AXL*, *CD74*, *CLEC7A*, and *CYBB*) (43). It may be that Toll-like receptor 4 regulates the formation of this cluster (43). Intriguingly, there was very little overlap between this microglial signature and others described (43) (**Figure 1**), with the exception of an E14.5 microglial cluster identified in a previously mentioned study (26).

2.3.8. White matter–associated microglia. White matter–associated microglia (WAM) were identified as a subset of $CD11b^{+}CD45^{+}$ cells from aged mice (18–20 months old) in white matter (corpus callosum, optical tracts, and medial lemniscus) versus gray matter from the frontal cortex (52). WAM were characterized by an upregulation of transcripts associated with lipid metabolism and the phagosome (*APOE*, *CD63*, *CLEC7A*, *CST7*, *LPL*, and *LYZ2*), cathepsins (*CTSB*, *CTSS*, and *CTSZ*), and MHC class II (*H2-D1* and *H2-K1*) and a downregulation of homeostatic genes (*CSF1R*, *CX3CR1*, *HEXB*, *P2RY12*, *P2RY13*, and *TMEM119*) (52). Putative WAM in situ ($IBA1^{+}$ cells in nodules of the corpus callosum) were able to internalize myelin (52). The presence of WAM in white matter and their ability to engulf myelin from aged brains were dependent on TREM2, as with other states, such as DAM (52). Safaiyan et al. (52) then investigated whether WAM and DAM were different states, and found that, despite overlap in some parts of their transcriptional signatures, WAM and DAM coexisted in mouse models of AD pathology ($App^{NL\text{-}G\text{-}F}$ and APP/PS1-$Apoe^{-/-}$), and, furthermore, WAM were not dependent on APOE signaling. The authors postulated that WAM and DAM may represent a continuous state, although in the work first identifying DAM, the intermediary state was not dependent on TREM2, unlike the WAM transcriptional signature (52). Potentially, a similar microglial state could be further differentiated based on the context, as WAM are more present in aging whereas DAM are present in AD pathology.

3. LIMITATIONS

The above studies highlight a number of defined microglial transcriptional signatures that are found during development and in disease conditions. This work demonstrates the diversity of microglia, particularly in their gene expression, and may point toward differences (and similarities) across ages, sexes, and regions, as well as within and between disease conditions. Partly because this field is relatively nascent, there remain a number of limitations and future directions, some of which are discussed in the following sections.

3.1. The Lack of Single Markers Versus Terminology

No single marker exists that either is specific to all microglia or differentiates microglia from other similar cell types, such as peripheral monocyte–derived macrophages. While this can make exclusive and complete assessment of the entire microglial population difficult, it highlights an opportunity to potentially target microglial states or subsets (or other macrophage states or subsets) with a particular transcriptional signature, particularly if their function(s) (and which transcripts may contribute to these functions) are further identified.

Furthermore, while the use of acronyms to refer to specific microglial signatures, clusters, and states can serve as a potentially helpful shorthand, it may also limit scientific advancement, in the same way that other simplistic categories, which are now outdated, did (e.g., resting versus activated and M1 versus M2) (49). It may imply a lack of overlap in transcripts across named microglial signatures, clusters, or states, which is not the case for all transcripts (**Figure 1**). The use of simplistic nomenclature may also inspire the idea that these are now fixed states of microglia, when it is unclear whether these represent irreversible transitions of microglia. Furthermore, microglia are incredibly plastic and may dynamically transition in and out of given states, depending on the context; therefore, the use of longitudinal techniques, such as in vivo two-photon imaging with fate mapping, would allow for the tracking of cells over time (4, 63).

3.2. Limitations of Data Sets

The work outlined in this review provides robust transcriptional information. As articulated in the studies cited herein, there are potentially robust regional variations in microglial transcriptomics (5, 21, 24), building on previous work showing differences when assessing microglia in situ and ex vivo, using other approaches, such as density, morphology, and trophic factor and immune mediator release (14). While these investigations may show regional variability (5, 21, 24, 38), they still are limited, as there is also subregional variation in microglia (e.g., in the hippocampus) (14). Furthermore, as other nervous system cells exhibit variations (e.g., neuronal and glial cell subtypes) across and within regions, understanding alterations in microglia in relation to these cell types is important. Of course, there are experimental concerns, as either more animals or cells would need to be utilized for smaller subregional analysis or the depth of sequence reads would be reduced. Therefore, the balance of cell numbers to be sequenced versus the depth of sequencing is an experimental and financial practicality. Utilizing techniques for in situ scRNA-seq, for example, will help resolve spatial dynamics as these approaches become more widespread.

Additional concerns arise from processing-induced alterations when generating single-cell preparations (44). The use of enzymatic dissociation, while increasing cell yield, has been demonstrated to alter a number of transcripts, including immediate early genes (*FOS* and *JUN*) and others associated with cellular stress response (*DUSP1* and *HSPA1A*), transcription regulation (*HIST1H1D* and *HIST1H2AC*), and immune signaling (*CCL3*, *CCL4*, *NFKBID*, and *NFKBIZ*). In situ RNA-seq could also help alleviate concerns about ex vivo processing that could be altering transcripts.

For human samples, there are also limitations in the described studies. Many of the studies highlighted herein utilize small sample numbers isolated from surgical resections, which could be potentially biasing because of a lack of heterogeneity in a small sample size and a localization close to pathology. A recent study has demonstrated that ancestry contributes to variation observed in microglia in the adult steady state (80). Therefore, potentially allelic information that could provide insight into function or treatment, particularly in the context of disease or injury, might be missed with small sample numbers.

3.3. Models to Humans

That much research has illustrated a high degree of similarity between mouse and human microglial cells, such as with enhancer regions and transcription factors associated with microglia (20), is promising; however, this similarity is not always the case for gene expression. Furthermore, predictive ligand–receptor pairs highlighted *IGF1* and *PTDSS1* as important for influencing the human fetal microglia transcriptome, whereas in mice, *APOE*, *BMP7*, and *CSF1* were found to be important embryonically. In addition, in humans, in contrast to mice, these same ligands were predicted as important for generating the postnatal transcriptome (27). Additionally, microglial *IGF1* was predicted as important for both embryonic (mouse) and fetal (human) microglial development, while microglial *PDGFB* and *TNF* were important for postnatal development (27). This may indicate that between humans and mice, different signals received by microglia result in the recruitment of similar pathways. More research is needed to interrogate these similarities and differences.

3.4. Transcript Versus Protein Versus Function

A major challenge that remains in the field is to clarify the functional and disease implications of the various markers and signatures of microglia, as highlighted in a recent consensus paper from the microglial field (49). Therefore, it is an imperative for the field to further use complementary proteomic approaches to validate whether changes in transcripts lead to changes in protein levels. The field should then further work to understand how these differently altered transcripts and proteins lead to functional changes in microglia that could have impacts on normative brain development and homeostasis but also disease and injury conditions.

Another limitation is the sheer number of transcripts typically altered between different clusters or signatures or between experimental groups. Certainly, it remains an issue of data management and visualization to highlight all of the transcripts that are altered, especially when they number in the hundreds or more, but selecting only a fraction to display could lead to primarily highlighting a subset of transcripts that are already of interest or commonly known to be altered in the field, while others are relegated to supplemental information tables. This can also bias the identification of differences versus resemblances between microglial signatures across studies and conditions and constitute an obstacle to future discovery. While comprehensive sets of data are typically available for subsequent analysis, less dense and easily searchable fieldwide databases, where multiple groups' findings are deposited, could facilitate future work, especially when seeking to look across regions, timepoints, and disease models, for example. Furthermore, pathway analysis may provide insight into the function of these microglial transcripts but is not a test of function in itself, especially depending on whether the analysis is based on identified function in microglia versus other similar cell types. Functional validation using in situ and in vivo approaches is thus required.

Recently, an article highlighted that microglia from juvenile mice, like many other cell types, particularly polarized ones, perform local translation in processes, particularly processes

associated with synapses (73). The findings that different subcellular compartments in microglia possess messenger RNA (mRNA) to engage in local translation point to another limitation, depending on the type of RNA-seq approach (75) (**Table 1**). Indeed, studies have shown differences between transcripts expressed in the whole cell versus the nucleus in human microglia from surgical resection samples (68). While the majority of transcripts were expressed similarly between compartments, there were a number of transcripts downregulated in the nucleus compared to the whole cell, including those associated with signatures identified in diseases, such as *APOE* and *SPP1* (68). Furthermore, ribosome-associated mRNA enriched in microglial processes included *APOE*, *C1QB*, *CD68*, *CSF1R*, *CTSS*, *P2RY12*, *SPARC*, and *TREM2* (73), which are found throughout different translational signatures (**Figure 1**). This research highlights increasing complexity in assessing microglia transcriptomics, with the addition of subcellular location.

4. FUTURE DIRECTIONS

4.1. Linking Morphological Phenotypes to Transcriptional Signatures

A key area for exploration is linking states determined transcriptionally with those observed using in situ and in vivo imaging modalities, such as foamy (22) or dark microglia (9, 62). As an example, dark microglia are positive for a number of markers (e.g., TREM2) that are transcriptionally found in some disease-associated signatures (e.g., DAM, MgND, ARM, and WAM) and downregulate a number of homeostatic markers (e.g., CX3CR1, IBA1, and TMEM119) (9, 62). Even though morphology, in the same way as transcriptomics, can lead to inferences and hypotheses regarding function, some methodologies used to determine these morphological states can provide insights about function. Ultrastructure analysis, which led to the discovery of dark microglia (9), for example, can assess phagocytic inclusions, and in some cases determine the nature of these inclusions (e.g., inhibitory or excitatory synapses and myelin) (17, 69, 71). Utilizing experimental approaches, such as correlative light and electron microscopy (CLEM) or array tomography, can help link microglial states observed using different techniques (56), potentially providing insights into function and helping to generate hypotheses for future work. Furthermore, the use of in vivo imaging, such as functional magnetic resonance imaging (MRI), which can be utilized in both model organisms and humans, can provide information longitudinally, which can then be combined with postmortem CLEM, to link changes on a macro level (e.g., functional connectivity between regions and volume increases or reductions among specific regions) to the underlying regional variations in brain ultrastructure (23).

4.2. Targeting for Monitoring and Treatment

The use of microglia-specific scRNA-seq data combined with transcriptome-wide association studies of diseases, such as AD, can not only provide insight into functional implications and therapeutic targets but also help develop novel analysis tools, such as radiotracers for positron emission tomography (PET), which can be utilized in both model organisms and humans—ideally, with specificity against particular microglial states or even cellular mechanisms (6). A recent article outlined a workflow that could be utilized to develop targets by first identifying microglial genes found across multiple scRNA-seq studies and, second, finding genes with increased expression in multiple studies of postmortem brains of people with AD, and then refining potential targets based on increased expression in microglia (i.e., a match of both) (6). From there, targets were prioritized based on a combination of the percentage of microglia that express the target gene, change in the target in AD versus nondisease controls, association with disease characteristics (e.g., plaque, tau, and cognitive scores), and druggability (6). While, for imaging, having a target

that is present on a lot of cells may increase feasibility, this approach would potentially limit the imaging of disease-related states, which could have important functions requiring selective modulation. Furthermore, similar approaches could possibly be utilized to discern potential promoters of interest for developing Cre-mediated viruses (40) and transgenic animal lines (12, 18) or other genetic targeting strategies (e.g., CRISPR-Cas9 systems) (72).

5. SUMMARY

Here, we have outlined recent work detailing our understanding of the transcriptional alterations in microglia, from embryonic development through early postnatal development into adulthood and aging and across contexts of health and disease. We discussed the resemblances and differences between microglial signatures identified so far and highlighted the importance of understanding the functions of the different states of the cells represented by the transcripts. We also highlighted current limitations of the data generated. Finally, we commented on potential future directions for the field. It will be critical moving forward to link the microglial transcriptional signatures identified to the morphological states that were observed in situ and in vivo and to further combine postmortem analyses with longitudinal studies involving MRI and PET in animal models and humans. Considering the essential functions performed by microglia across the life span, both physiological and immunological, selective monitoring and targeting of their states using new tools providing selectivity will be critical to unravel how to distinctively modulate their contextually relevant functions.

FUTURE ISSUES

1. Transcriptional analysis provides potential insight into functional pathways, but future studies should further investigate the importance of transcript alterations with regard to microglial functions.
2. Investigating genes similarly and differentially altered across signatures could provide insights into the regulation of microglial functions that are similar or different across disease conditions.
3. Many of the altered transcripts point toward lipid processing; therefore, future work should incorporate lipidomics into workflows to gain a clearer insight into potential functions.
4. The development of field-wide repositories that are easily searchable along region, disease, or transcript, for example, could facilitate more hypothesis-driven, functional analysis.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

We acknowledge with respect the Lekwungen peoples on whose traditional territory the University of Victoria stands, and the Songhees, Esquimalt and WSÁNEĆ peoples whose historical relationships with the land continue to this day.

H.A.V. is supported by a fellowship from the Canadian Institutes of Health Research and is a Michael Smith Health Research BC Research Trainee. M.-È.T. holds a Canada Research Chair (Tier 2) in *Neurobiology of Aging and Cognition*.

We acknowledge the following Tremblay laboratory members for their thoughtful review of the manuscript: Adriano José Maia Chaves Filho, Chloe McKee, Colin Murray, Katherine Picard, and Eva Šimončičová. We apologize to the many researchers whose work could not be appropriately cited due to space constraints.

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