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Transcriptional and epigenetic regulation of microglia in health and disease

Hana Yeh[1,2] and **Tsuneya Ikezu**[2,3]

[1]Graduate Program in Neuroscience, Boston University School of Medicine, Boston, MA 02118

[2]Department of Pharmacology and Experimental Therapeutics, Boston University School of Medicine, Boston, MA 02118

[3]Department of Neurology, Boston University School of Medicine, Boston, MA 02118

Abstract

Microglia are the resident immune cells that maintain brain homeostasis and contribute to neurodegenerative disorders. Recent studies of microglia at transcriptomic and epigenetic levels revealed specific molecular pathways that regulate microglia development, maturation and reactive states. The transcriptionfactor PU.1 plays a key role in regulating several microglial functions. Environmental factors such as microbiota, early life stress and maternal immune activation can dysregulate PU.1 and innate immune response. This review discusses the epigenetic regulation of key transcriptional factors in human and murine microglia, highlighting their networks for shaping the microglial function. PU.1 and other microglia-specific transcriptional factors can be further studied to determine their therapeutic applications in neurologic disorders.

Keywords

Alzheimer’s disease; autism spectrum disorders; early life stress; epigenetic; microglia; neurodegenerative disorders; PU.1; transcription factors

The transcriptomic and epigenetic landscape of microglia

Microglia are the resident immune cells that maintain homeostasis in the central nervous system (CNS). Microglia share several markers with monocytes, but are derived from the embryonic yolk sac and have distinct lineage from bone marrow-derived myeloid cells [1]. Microglia support neuronal functions and networks in addition to maintaining brain homeostasis [2]. Under physiological conditions, microglia actively monitor their surroundings, respond to perturbations, clear cell debris, and regulate synaptic transmission [3]. During development, they modulate synapse formation and elimination, revealing

Corresponding authors: Hana Yeh (hyeh@bu.edu) and Tsuneya Ikezu (tikezu@bu.edu), 72 East Concord St, L-606, Boston, MA 02118.

microglia's role in shaping the brain circuitry [4]. Microglial dysfunction contributes to the pathogenesis of neurodegenerative disorders including Alzheimer's disease (AD) and neurodevelopmental disorders including autism spectrum disorder and schizophrenia [5-7]. In neurodegenerative state, microglia can enhance neuroinflammation leading to more cell deaths [6, 8]. Although not as clear in neurodevelopmental disorder, microglia can have impaired pruning or secrete molecules that contribute to hyperactivity in the CNS [7, 9]. This further emphasizes the need to define the molecular mechanisms that control microglial functions. Investigation on how microglial function is regulated at transcriptome and **epigenome** (See Glossary level is invaluable in developing therapeutic strategies for neurological disorders.

Advancement in defining microglia-specific markers has enabled scientists to sort, characterize and determine the molecular phenotype of microglia at different states. RNA-sequencing studies have identified microglia-specific genes such as olfactomedin-like protein 3 *(Olfml3)*, which is involved in early development patterning, and sialic acid binding Ig-like lectin H *(Siglech)*, which is involved in innate immune cell differentiation [10]. Microglial markers such as purinergic receptor P2Y G-protein coupled 12 *(P2ryl2)*, transmembrane protein 119 *(Tmem119)*, and Fc receptor-like S, scavenger receptor *(Fcrls)*, are used to define microglia homeostatic state. Whereas, inflammatory marker *Clec7a* is used to define microglia in neurodegenerative state [11]. Transcriptomic analysis of isolated mouse microglia revealed that microglia express a unique genes sets called the "sensome" that enable them to sense endogenous ligands and interact with their environment [12].

In mice, microglia undergo a step-wise program of maturation that is synchronized with the developmental phases of the brain [13, 14], which consists of three distinct temporal stages: early microglia spanning until embryonic day 14, premicroglia from embryonic day 14 to a few weeks after birth, and adult microglia from a few weeks after birth onward [13]. The transcriptomic profile can be shaped by intrinsic factors, such as age and sex, pathogenic and tissue-specific cues including protein aggregates, stress signals, nutrients, and external cues such as signals from the microbiota colonizing gastrointestinal and urogenital tracts [15]. Under pathological circumstances, cues such as debris or stress signals from surrounding tissue can lead to transcriptomic and morphological changes in microglia, which can switch from homeostatic to reactive states [16, 17]. Here, this review is focused on summarizing the recent discoveries on the molecular mechanisms of how environmental cues can affect microglial function.

Elucidating critical transcriptional regulators of microglial gene expression

The list of known microglia-specific genes is constantly being updated based on improved microglial isolation techniques and labeling methods. Microglial transcriptomes are tightly regulated by multiple key **transcription factors** such as *Sall1*, *Spi1*, *Mafb*, and *Mef2c* [5, 15]. Recent genomic and proteomic studies revealed microglia express a unique set of genes distinct from infiltrating monocytes and other immune cells [18, 19]. Barres group recently identified *Tmem119*$^{++}$/*Sall1*$^{+}$/*Gpr56*$^{+}$/**Apoe**$^{+}$ as yolk sac-derived microglia, whereas **hematopoietic stem cell**-derived microglia-like cells express *Tmem119*$^{+}$/*Ms4a7*$^{+}$/*Clec12a*$^{+}$/*Apoe*$^{+++}$ suggesting lineage-specific microglia markers that can be useful in determining the

origin of microglia [18, 19]. In addition to microglia, new $CD38^+$ resident myeloid cells called CNS border-associated macrophages (BAMs) are embryonically-derived cells that line along the meninges, choroid plexus, and perivascular spaces [20]. Both microglia and BAMs are $CD11b^+/CD45^{low}$, making it difficult to distinguish between them without localization patterns. Based on current **FACS** and immunohistochemical analysis, the transcription factor SALL1 was identified as a key marker for microglia. High expression of SALL1 in microglia allows distinguishing $SALL1^+$ microglia from $SALL1^-$ BAM cells and infiltrating peripheral monocytes [20, 21]. Currently, FACS and immunocytochemistry are used to distinguish microglia, macrophages and BAM based on RNA-seq identified gene markers including differential expression of CD11b, CD45, CD38, CLEC7A, FCRLS, P2RY12, SALL1 and TMEM119.

In addition to FACS and immunohistochemical approaches, **assay for transposase accessible chromatin sequencing** (**ATAC-seq**) can evaluate transcriptional networks that control microglial signature at an epigenetic level determining the chromatin accessibility landscape in microglia [5, 22, 23]. Using this approach, Gosselin et al. reported PU.1 transcriptional network as the most abundantly expressed **ETS domain transcription factor** in both murine and human microglia [22]. PU.1 is an ETS-domain transcription factor encoded by *SPI1* (human) or *Spi1* (murine) gene. PU.1 binds to purine-rich sequence (PU-box) and activates gene expression during immune cell development [24, 25]. Microglia development is largely controlled by the transcription factors IRF8 and PU.1. The microglia-enriched PU.1 binding regions were also enriched with *Smad*, *Mef2*, and *Ctcfl* family-**binding motifs**, identifying interactions between the transcription factors [5]. Furthermore, four binding motifs *Mafb*, *Stat3*, *Usf1*, and *Smad2* were preferentially associated with PU.1 binding specifically in microglia in mice, suggesting these transcription factors are microglia-specific co-regulators that cooperate with PU.1 for establishing microglia-specific gene **enhancer** profiles [5].

The role of PU.1 in the maintenance of homeostatic microglia

Spi1 (PU.1) mRNA is continuously expressed from EMPs to adulthood microglia, suggesting the necessity of PU.1 for microglial development and functional maintenance [1]. **Chromatin immunoprecipitation (ChIP)-seq** analysis of immortalized mouse microglial cell line BV2 cells revealed that about 63% of microglial sensome genes are PU.1 targets, suggesting that PU.1 regulates specific microglial functions [26]. PU.1-deficient mice display multiple immune-developmental abnormalities, including parenchymal microglia [27]. Accordingly, PU.1-deficient mice have reduced expression of *Csf1r*, *Csf2r* and *Csf3r*, suggesting a critical role of PU.1 for the expression of essential cell survival receptors [28].

Alterations in the PU.1 expression level can change microglial morphology and function. PU.1 expression levels are similar in isolated human glial cells from biopsy and postmortem adult human brain tissues [29]. However, silencing of PU.1 by siRNA reduced the number of microglia and their ramification and phagocytosis of amyloid-beta peptide 1-42 (AB42) in mixed glial culture in their study [29]. Silencing of PU.1 in human microglia culture suppressed expression of myeloid adapter protein *DAP12/TYROBP,* ***TREM2****, LST1, CD11b, MRC1, CEBPA, PTPRC* and MHC class II genes *HLA-DR, HLA-DP* and *HLA-DQ*

[30, 31], suggesting its role in antigen presentation and pro-inflammatory response by PU.1 [30, 31].

Beside PU.1 as a key transcription factor, other transcriptional regulators are involved in the precise tuning of microglial homeostasis, making microglial transcriptome tightly regulated (Table 1). These molecules are necessary to understand how microglial transcriptome can be regulated in various states. *In vitro* and *in vivo* studies show that PU.1 act as a master regulator and controls the development and maintenance of homeostatic function in microglia, such as CSF1R-mediated cell survival, phagocytosis, antigen presentation, and ramified morphology, but has no effect on **TLR4**-mediated inflammatory responses. But further research is required to determine how these different transcription factors interact to maintain microglial identity and determine if there are differences between human and murine microglia.

Epigenetic regulation of transcription factors in microglia

Immune memory in microglia and macrophages are shaped by epigenetically mediated alterations in the enhancer repertoire of targeted genes [11, 32]. Microglial gene enhancers form complexes with **lineage-determining transcription factors** including PU.1 and SALL1, and become co-activators to regulate the transcription of targeted genes [33]. ATAC-seq of isolated microglia across different developmental stages showed that accessibility of **promoter regions** by transcriptional complexes was largely conserved over time [13, 34]. Acetylation of histone H4 on the promoter and intron-1 region of the PU.1 locus are tightly regulated for allowing the interaction between the locus and RNA polymerase II. Inhibition of **histone deacetylase** (**HDAC**) increases acetylation of H4 and disrupts the interaction between the locus and RNA polymerase II, leading to the suppression of PU.1 transcription in murine microglia [35, 36]. Valproic acid treatment, which selectively inhibits the catalytic activity of class I HDACs and induces proteasomal degradation of HDAC2, of primary adult human microglia reduces phagocytic capacity and expression of PU.1 and CD45, suggesting the its regulation of microglial phagocytosis via epigenetic mechanism [36-38]. Furthermore, Rustenhoven et al. identified another HDAC class I/II/IV inhibitor vorinostat as an effective attenuator of PU.1 in primary human microglia-enriched culture [30, 39]. However, vorinostat treatment showed reduction in *DAP12* expression, whereas expression of *CD45* and *HLA-DR*, *HLA-DP* or *HLA-D1* remained unchanged [30]. This discrepancy could be due to the difference in the coverage of HDAC classes or off-target effect of the drugs. Studies with HDAC inhibitors show that microglial transcriptome can be epigenetically regulated, possibly through PU.1 suppression. More precise epigenetic studies are necessary by genetic targeting of specific HDACs in microglia.

Several groups have identified epigenetic regulators that can contribute to microglial function (Table 2). **Methyl-CpG binding protein 2** (**MECP2**) regulates microglial responsiveness to environmental stimuli. MECP2-deficiency, as seen in Rett Syndrome, can lead to microglial activation and dysfunction [17]. Global loss of *Mecp2* leads to reduced numbers of microglia, perivascular meningeal macrophages, intestinal macrophages and $LY6C^{lo}$ circulating monocytes [17]. MECP2 acts as a microglia-specific transcriptional

repressor of SNAT1, a major glutamate transporter. MECP2-deficient microglia have impaired glutamate synthesis and mitochondria function [40]. Further examination with ChIP-seq analysis revealed that Mecp2-deletion increased histone acetylation at enhancer regions of *Fkbp5*, a canonical glucocorticoid target gene, and recruitment of nuclear receptor co-repressor2 (NCOR2) and HDAC3 complex [17]. These results show dysregulation of genes involved in glucocorticoid signaling, hypoxia response and inflammatory responses, suggesting *Mecp2* is critical for maintaining immune cell function including microglia [17].

Histone H3 at lysine 4 (H3K4) is used to package DNA in eukaryotic cells including human cells, and modifications to the histone alter the accessibility of genes for transcription. Recent ChIP-seq results have revealed active H3K4me2 regions shared in both MGnD and wild-type microglia, suggesting that the disease-associated regions primed in MGnD can be primed even in homeostatic microglia [41]. Peripheral immune stimulation and cerebral beta-amyloidosis can lead to epigenetic modification in microglia. Cerebral beta-amyloidosis activates HIF-1a and mTOR pathways, which leads to transcriptional and functional alterations associated with increased inflammatory and immune response genes in MGnD [11]. In addition, APP mice with four-time LPS treatment showed increased H3K4me1 levels in putative enhancers related to phagocytic function [11]. These data suggest the differential effect of immune training versus tolerance due to multiple peripheral immune stimulation, which is reflected in the epigenetic landscape of MGnD in AD mouse models. Further research can reveal how other stimuli may lead to chronic modulation of microglia response and contribute to the progression of the neurodegenerative disorder.

Microglia can efficiently clear apoptotic cells and debris, which is regulated by H3K27me3, a histone methylation occurring on the amino (N) terminal tail of the core histone H3. This tri-methylation is associated with the downregulation of nearby genes via the formation of heterochromatic regions. In the adult brain, clearance activity of microglia is suppressed in areas with low frequency of neuronal deaths [42]. This region-dependent phagocytic activity is regulated via suppression of clearance-related genes by epigenetic modification of H3K27me3 based on ChIP-Seq analysis. In contrast to striatum microglia (stMG), cerebellar microglia (cbMg) displayed a phagocytic microglia phenotype with reduced ramifications and cell volume that is characteristic of immature microglia and MGnD, but lack enrichment of pro-inflammatory genes in mice [42]. Single-nuclei RNA-seq and proteomic analysis validated that mouse cbMg expressed higher levels of clearance-associated proteins such as AXL, LC3, APOE, CD74, MHCII, and MRC1. These studies demonstrate the epigenetic regulation of clearance-associated genes for the regulation of microglial phagocytosis in a brain region-specific manner.

In addition to histone modifications, microRNAs (miRNAs) are also involved in controlling the epigenetic landscape in the CNS. miRNAS are small non-coding RNA molecules involved in silencing and post-transcriptional regulation of gene expression via base-pairing with complementary sequences within mRNA molecules. Through miRNA and mRNA microarray assays, gene regulators such as miR-124 and miR-155 have been identified in microglial activation pathways [24, 43]. Several miRNAs including miR-124 and miR-155 modulate the development of microglial cells. miR-124 is suggested to directly inhibit C/EBPa and PU.1 to prevent acquisition of a reactive microglia state [44]. These studies

suggest that miRNAs including miR124 and miR155 act as key regulators of microglia quiescence in the CNS.

Microglia in neurodegenerative state

In diseased states, microglia can become dysfunctional and neurotoxic, which are referred as disease-associated microglia (DAM) or microglia neurodegenerative phenotypes (MGnD). DAM or MGnD are found in neurodegenerative disorders including AD, amyotrophic lateral sclerosis (ALS) and multiple sclerosis [41, 45]. In AD brains, microglia surrounding amyloid plaques display activated amoeboid morphologies with enlarged soma and retracted processes, indicating microglial response to protein accumulation or increased inflammatory mediators [41, 46-48]. In mouse models of AD such as amyloid precursor protein (APP)/ presenilin-1 (PS1) and 5XFAD transgenic mice, there is a subset of microglia surrounding amyloid plaques with elevated phagocytic and microglial activation markers including Galectin-3 and CLEC7A, and reduced expression of homeostatic markers such as P2RY12. This suggests a switch from homeostatic to reactive microglial state in specific population associated with amyloid plaques [46]. Krasemann et al. found **dystrophic neurites** stimulate TREM2 –APOE pathway that results in phenotypic switch from homeostatic to MGnD microglia [45]. Customized NanoString-based gene expression analysis, which uses molecular barcodes and microscopic imaging to detect and count unique transcripts in hybridization reactions, revealed APOE and TGFβ as the major upstream regulators of MGnD microglia. Upregulation of *APOE* suppresses gene expression of microglial homeostatic transcriptional factors including *Spi1*, *Mef2a*, *Mafb*, *Smad3*, and induces inflammatory response via induction of transcription factors *Bhlhe40*, *Tfec*, *Atf*, and inflammatory miRNA *miR-155*, which if genetically ablated, can reverse abnormal microglia signature and ameliorate disease in SOD1 mice, a mouse model for ALS [12, 45]. More recently, Litvinchuk *et al.* also identified microglia-specific transcription factors *Irf8*, *Spi1*, *Runx1* are significantly upregulated in FACS-isolated microglia in PS19 mice, a model for neurodegenerative tauopathy and Alzheimer's disease [49]. Deficient-*C3ar*, the complement factor C3 complement receptor that mediates neuroimmune crosstalk critical in network function, reduces expression of *Irf8*, *Spi1*, and *Runx1* in PS19 mice [6, 49, 50]. This novel finding shows the detrimental effect of activated complement system and how expression of microglial transcription factors can be recovered by inactivation of a complement pathway in a neurodegenerative model [49]. Through unbiased approach using RNA-seq or Nanostring assays, specific pathways such as TREM2-APOE and C3-C3AR can be identified to determine critical regulators in neurodegenerative state in microglia. This approach also identifies key gene markers to identify sub-populations of microglia associated with disease-states, which can be further tested in additional mice models including aging.

PU.1 also plays a role in traumatic brain injury (TBI). Genome-wide methyl binding domain (MBD)-seq and RNA-seq of brain tissues in TBI induced by lateral fluid percussion in adult male Spraque-Dawley rats showed molecular networks and mechanism underlying the chronic dysregulation [51]. Transcription factors including *Spi1*, *Cebpd*, *Pax6*, *Tp73* were upregulated at 3 months after TBI [51]. Together, these studies imply that PU.1 and other

transcription factors are critical in maintaining homeostatic microglia transcriptome, but can be influenced by neurodegenerative disorder or TBI.

Microglia transcriptome in aging

Transcriptome analysis of aged human microglia reveal specific genes influenced by aging [52-54]. With maturation and aging, expression of pro-inflammatory genes in adult microglia increase, suggesting their reactive state and reduced plasticity in the adult stage possibly due to repetitive exposure to infectious agents and active removal of cell debris [53, 55]. In aging mice, the microglial sensome transcripts for sensing endogenous ligands including *Trem2*, *Gpr34*, *P2ry12*, *Fcrl1*, and *Dap12*, are downregulated. Microbe recognition and host defense genes such as *Tlr2*, *Cd74*, *Ltf*, *Clec7a*, *Cxcl16*, are also upregulated [12]. Interestingly, Wehrspaun et al. analyzed three transcriptome data sets from postmortem human cortical tissue spanning teens to elderly, and found microglial markers to be assembled into a gene co-expression network regulated by an age-dependent transcriptional module consisting of *RUNX1*, *IRF8*, *SPI1*, and *TAL1* [53]. Further analysis revealed that microglia surface receptors for microglia-neuron crosstalk (*CX3CR1*, *P2RY12*, and *TREM2*) and TLRs for communication with pathogen-associated molecular patterns tended to show a negative correlation between gene expression level and age, highlighting the age-dependent change in microglial plasticity [53, 55]. More recently, Olah et al. suggested a protective effect of *APOE2* haplotype against increased expression of aged human microglia gene set in human microglia [12, 56]. Their results demonstrated upregulation of *CD33*, *INPP5D*, *MS4A4A*, *SORL1*, and *TREM2* in aged microglia, but no change in PU.1. Compared to previous reports, the mean age of the postmortem tissue donors was greater at 95-years old and their cohort consisted of mostly females, which could explain the discrepancy [56]. However, lack of PU.1-enrichment in aged human microglia gene set might also be due to tissue processing differences including postmortem tissue degradation or microglial isolation. In summary, aged microglia show reduced expression of sensome genes and increased expression of neuroprotection genes, which are modulated by RUNX1, IRF8, PU.1 and TAL1, supporting aged microglia should be not considered dysfunctional but rather have shifting functional roles in aged brain to maintain brain circuit function.

The effect of microbiome on microglia transcriptome

The gut microbiome has emerged as a key regulator of the immune system and microglial physiology. Accumulating evidence indicates that PU.1 expression significantly alters the microglia phenotypes via reshaping the gut microbiota landscape. Microglia are dysregulated in mice with dysbiosis, either due to microbial imbalance or maladaptation [15]: gut symbiosis promoted the maintenance of microglia under homeostasis conditions, while germ-free (GF) condition led to impaired microglia maturation, differentiation and function [15]. RNA-seq analysis of microglia from GF mice revealed that microglia transcription factor PU.1 and survival factor CSF1R were significantly upregulated in microglia from GF mice [1, 15]. The immature phenotype of GF microglia could be corrected by microbiota recolonization [15]. However, the mechanism in which microbiota can influence the maturation and homeostatic state maintenance remains to be determined. These research findings based on mouse models can be helpful in providing shared

mechanisms between human and mice on how microbiota can influence microglial phenotype and transcriptome. The human microbiota can be more complex due to changing diet and activities compared to laboratory mice microbiota.

ATAC-seq analysis of embryonic microglia from specific pathogen-free (SPF) and GF mice have shown that several regions of the differentially accessible regions are more accessible in embryonic microglia of SPF but not GF mice during development [57]. More recently, Thion et al. demonstrated that microglia acquire sexually dimorphic transcriptome profiles and different response to the GF environment in C57BL/6J SPF and GF mice [57]. Guneykaya et al. also reported sex-dependent differences in transcriptomic and proteomic profiles in microglia [58]. However, PU.1 was not among the top transcription regulators, supporting the idea that PU.1 is a critical regulator in microglial identity and survival that can be regulated by the presence of microbiota in sex-independent manner. These studies suggest that gut microbiota can induce subtle, but global chromatin accessibility changes during embryonic microglia development and regulates several key targets involved in microglial maturation in sex and stage-specific manner.

Early life environmental perturbation of microglia

Maternal immune activation (MIA) by administration of polyinosinic:polycytidylic acid (polyI:C), which is a synthetic analog of double-stranded RNA used to simulate viral infections via interaction with TLRs, can disrupt pre-microglial development in offspring, leading to a more advanced developmental stage. Hippocampal microglial cells from polyI:C offspring mice showed dysregulation of genes associated with inflammatory response, cell migration and phagocytosis [13, 59, 60]. Microglia from polyI:C offspring had reduced expression of PU.1 and its target genes, which were restored after administration of anti-inflammatory drug minocycline [26, 59]. Downregulated genes included *Fcgr1*, *Itgav*, *P2ry6*, *Sirpa*, *Siglece* and *Cx3cr1* that constitute the sensome. This study demonstrates the significant environmental effect by maternal immune activation on microglial gene expression phenotype, which are regulated by PU.1, and show similarity to those of microglia derived from AD mouse models including APP/PS1 mice (Table 3). Future studies are needed to validate the function of PU.1 in dysregulated microglia in both germ-free and MIA mice.

More recent studies have linked microbiota with behavioral deficits associated with MIA [61, 62]. The MIA phenotypes require maternal intestinal bacteria that promote Th17 cell differentiation [63]. In addition, when polyI:C offspring are orally treated with human commensal *Bacteroides fragilis*, the gut microbiota composition is altered and ameliorates MIA phenotypes including behavioral abnormalities [61]. Other environmental factors such as early life stress may also affect microglia development. Early life stress caused by maternal separation can induce pro-inflammatory microglia phenotype in offspring and reduced PU.1 transcriptional activity [64]. Together, these studies suggest microbiota have a large impact on the host metabolome and behavior as observed in microglia development and MIA studies. Further research is necessary to identify additional environmental factors and determine how different combinations of factors can influence microglia development and homeostasis.

PU.1 in transcriptomic and epigenetic alterations *in vitro*

Microglia culture models have been developed to recapitulate microglia *in vivo*, but have multiple limitations. Microglia lose their distinct expression patterns once cultured *in vitro* [5, 22]. Gosselin et al. reported that majority of transcript levels between human and mouse microglia were similar, but there are genes with species-specific bias in expression magnitudes [22]. Expression levels for complement system genes such as C2 and C3 and brain structure developmental genes including *SYNDIG1* and *GLDN* were higher in human microglia, showing distinct differences between the human and microglia transcriptome [22]. Another limitation of microglia culture is that it develops immature microglia with amoeboid morphology, rapid proliferation, and heightened phagocytic activity [65]. It can be reversed by engraftment of isolated cells into brains lacking microglia, suggesting that additional CNS-specific cues are required to sustain microglia specification [18, 66].

Primary human microglia remain poorly characterized due to limited accessibility, lack of standardized isolation methods and culturing conditions. Recently, protocols for induce human iPSC-derived microglial-like cells (iMG) have been developed [67-71]. These protocols use induced pluripotent stem cell (iPSC) to differentiate them into iMG. iMG exhibited a transcriptomic phenotype more similar to human microglia *in vitro* than *ex vivo* microglia. (Table 4) [22, 67, 68]. iMG can integrate into organotypic 3D culture [67]. When co-cultured with iPSC-derived neurons, iMG display a more motile phenotype and dampened secretion of cytokines compared to monoculture [67, 68]. Numerous protocol used to culture microglia require complex recipes that induces key microglial regulators including PU.1 to recapitulate microglia *in vivo*. Further improvements are necessary for the differentiation of iPSC to more microglia-like cells for functional studies.

Most of the mRNAs encoding transcription factors that recognize enriched motifs in microglia enhancers exhibited significant reductions *in vitro* [22]. *De novo* analysis of ATAC-seq peaks associated with a significant decrease in H3K27ac-recognition elements for PU.1, IRF, CTCF, AP-1, SMAD, and MEF2 motifs, revealing epigenetic regulation. Tissue micro-environmental factors activate cascades resulting in co-operative binding of certain transcription factors with PU.1, leading to a unique enhancer profile enabling microglial dynamic response to its surrounding micro-environment [24, 72]. Several groups have attempted different cell culture conditions to maintain homeostatic microglia signature and ramified morphology *In vitro* using a variety of cytokines, such as CSF1, GM-CSF, TGFβ, and IL-34 [73-76]. Butovsky et al. showed TGFβ act as a crucial upstream regulator in microglia survival, differentiation and maintenance of microglia signature *In vitro* [24]. Further improvements such as overexpression of PU.1 in microglia to enhance CSF1R signaling can be considered as well in future studies [31]. However, the addition of key factors for microglial maintenance can hinder the interpretation of *In vitro* disease models, since factors such as CSF1 can promote microglia viability and increased PU.1 expression that can also influence microglial function in disease states [76].

Concluding remarks

The tight regulation of microglia transcription involves multiple key transcriptional factors including PU.1, SALL1, MEF2, and MAFB (Figure 1). Among these factors, PU.1 is one of the more specific factors critical to microglial identity and survival and is sensitive to micro-environmental cues. Current studies show that PU.1 can interact with the other specific transcription factors and epigenetic regulators such as HDAC, highlighting the importance of PU.1 in regulating microglial homeostatic and reactive state at transcriptional and epigenetic levels. Dysregulation of transcription factors in disease states are likely responsible for altering the microglial gene expression landscape, which may contribute to the disease progression. Identification of key transcription factors and epigenetic factors will lead to development of effective therapeutic strategies via modulation of MGnD in the future. Immune training and induction of immune memory of microglia can be potentially beneficial in suppressing their pro-inflammatory response in disease states. Additional studies will be necessary to address that (se Outstanding Questions), which would direct to a better understanding of how transcriptional regulators orchestrate homeostatic and neurodegenerative microglia.

RNA-seq and ChiP-seq provide powerful platforms to characterize microglia in homeostatic and neurodegenerative state, but face certain limitations (Table 5). Future work should focus on single-cell transcriptomics since the recent accumulation of data based on RNA-seq and ChIP-seq are dependent on bulk sequencing. Single-cell RNA and ChiP-sequencing can provide characterization of subpopulations, distinct transcriptomic phenotype, but may have significant limitation in sequencing depth. Focusing on individual cells across ages and brain regions to refine key regulators in different states and evaluate the variability in cell identity, response and location in the CNS may constitute the next level of microglial transcriptomic characterization (see Clinician’s Corner). Further research should also focus on the discrepancy between human and mouse microglia transcriptome regulation, which can provide targets that shared between mouse and human microglia for therapeutic strategies.

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Glossary

Apolipoprotein E (APOE):
A class of lipid-binding proteins involved in transportation and metabolism of lipids, lipid-soluble vitamins and cholesterol.

Assay for transposase accessible chromatin (ATAC) sequencing:
A method for mapping chromatin accessibility genome-wide using Tn5 transposase that inserts sequencing adapters into accessible regions of chromatin.

Binding motif:

A collection of DNA binding sites that can be represented by a consensus sequence in which are bound by DNA-binding proteins to regulate gene transcription.

Chromatin immunoprecipitation (ChIP)-seq:
A method to identify the genome-wide DNA binding sites for specifically modified chromatins by combining chromatin immunoprecipitation (ChIP) with DNA sequencing.

Dystrophic neurites:
Abnormal neuronal processes characterized by aberrant sprouting, dystrophic expansion, and accumulation of various cellular organelles and cytoskeletal proteins, such as microtubule-associated tau proteins.

Enhancer:
Enhancers are short regions of DNA that form complexes with transcriptional factors to regulate the transcription of targeted genes

Epigenome:
The study of change in phenotypes such as post-translational modifications of chromatins and DNA, which does not involve alteration in DNA sequence.

ETS domain transcription factor:
Characterized by the presence of a conserved DNA-binding domain and interacts with multitude of co-regulatory partners to elicit gene-specific responses and drive distinct biological processes.

Fluorescence-activated cell sorting (FACS):
Fluorescence intensity-based sorting for collection of specific cell populations

Hematopoietic stem cell:
A subset of stem cells found in peripheral blood and bone marrows that can develop into all types of blood cells.

Histone deacetylase:
A class of enzyme that removes the acetyl group from histone proteins on DNA, making the DNA less accessible to transcription factors.

Immune memory:
A cellular immune function for specific recognition of an antigen that the body has previously encountered. In microglia, there are two types of immune memory: training, which augments innate immune responses; and tolerance, which dampens them.

Lineage-determining transcription factor:
Transcription factors that bind to enhancer regions and recruit secondary transcription factors for cell-type-specific gene expression.

Methyl-CpG binding protein 2 (MECP2):
This protein is capable of binding to methylated DNA, further condensing the chromatin structure to repress or activate gene transcription depending on the context.

Promoter regions:
DNA sequences that define where transcription of a gene by RNA polymerase begins and initiates transcription of a particular gene

Toll-like receptors (TLRs):
Evolutionally conserved innate immunity receptors for detecting pathogen- and damage-associated pattern molecules for host-defense response.

Transcription factor:
A protein that controls the transcription from DNA to messenger RNA.

Transcriptomics:
The study of genome-wide set of all RNA molecules, including mRNA, non-coding RNA.

Triggering receptor expressed on myeloid cells 2 (TREM2):
A transmembrane molecule expressed in microglia and myeloid cells, which is functionally implicated in phagocytosis.

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Outstanding Questions:

- How do transcription factors work with one another to regulate microglia in different disease states at a single cell level?
- How can we develop therapeutic approach that target modulation of epigenetics via immune training in microglia?
- Which of the transcriptional factors are potential therapeutic targets for health and disease?
- What improvements can be made to study microglia *in vitro* to resemble *In vivo* mature microglia?
- How can microglia isolation methods and RNA-seq processing methods be standardized for reproducibility and comparison between datasets?

Highlights:

- Microglia transcriptome is tightly regulated by a combination of transcription factors, such as PU.1, for their role in the development and homeostatic function of microglia.
- Environmental factors including microbiome, maternal immune activation, and early life stress can influence the transcription factor network and epigenetic modification of target genes.
- Distinct clusters of transcription factors regulate the transcription network in early development, homeostatic and disease conditions
- The PU.1-regulated genes are influenced by epigenetic modifications, which are altered by disease and tissue culture environment

Clinician's Corner:

- Non-specific anti-inflammatory drugs such as minocycline can negate the balance of neuroprotective and neurotoxic function of microglia.
- Disease-specific microglial transcription factors are new therapeutic targets of neuroinflammation
- Complement system and microglia-specific immune molecules are novel therapeutic targets of neurodegenerative disorders.
- Analysis of disease-specific microglial markers will facilitate evaluation of the clinical outcome of anti-neuroinflammatory therapy
- The information obtained from murine microglia may differ from those from human microglia. The transcriptomic and epigenetic results will require careful validation using freshly isolated human microglia.

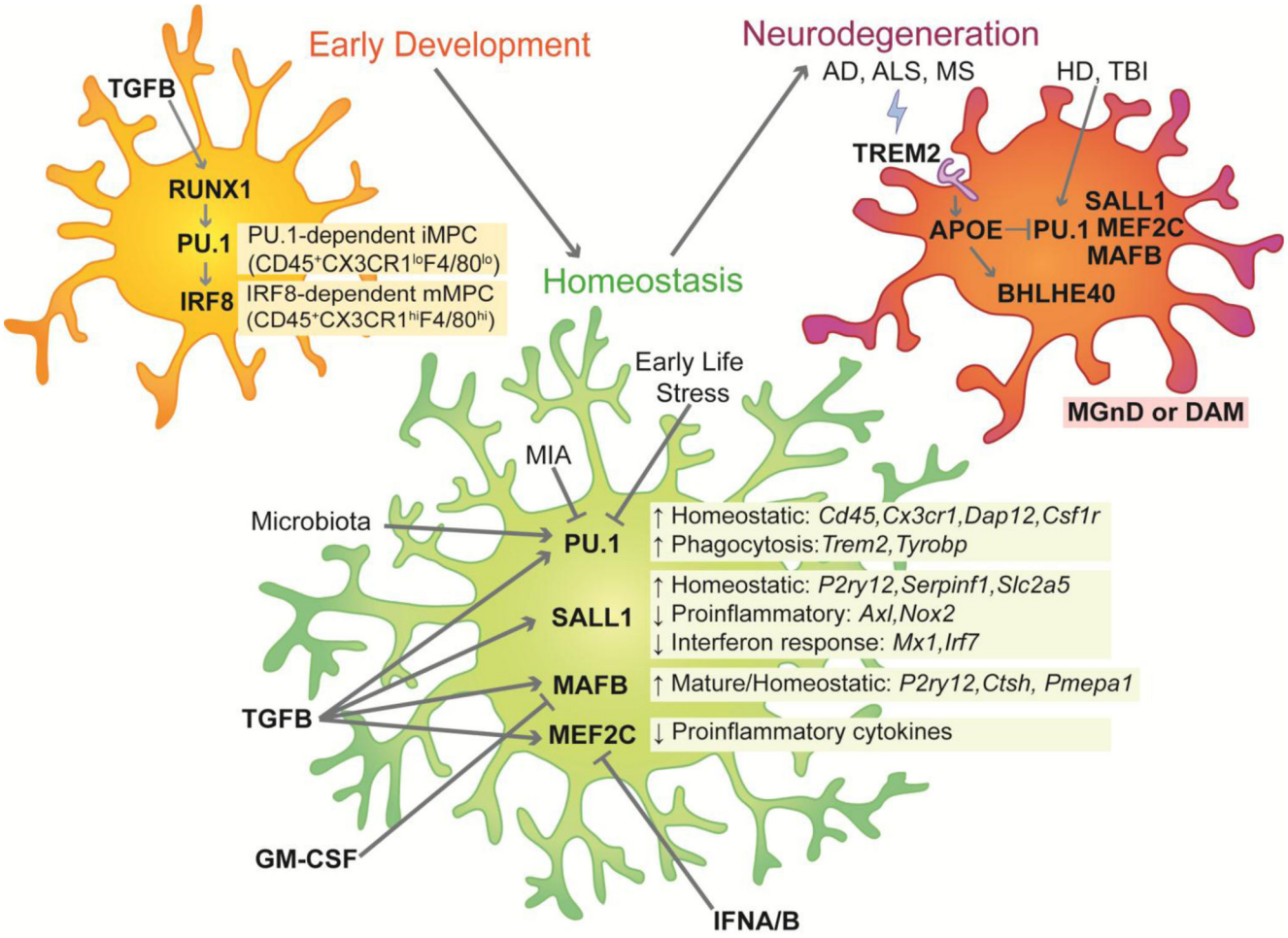

Figure 1: Key transcription factors in early development, homeostasis and neurodegeneration.

In early development microglia (yellow) the transcription factors RUNX1, PU.1 and IRF8 are very active. In homeostatic microglia (green) PU.1, SALL1, MAFB, MEF2c regulate certain homeostatic genes, external factors can epigenetically regulate the expression of these transcription factors. Neurodegenerative microglia (red), also referred as disease-associated microglia (DAM) or microglia neurodegenerative phenotypes DAM (MGnD) display increased signaling of APOE pathway via TREM2 (purple) that inhibits the expression of key transcription factors and activates BHLHE40 that is associated with the neurodegenerative state of microglia. (AD: Alzheimer's disease, ALS: amyotrophic lateral sclerosis, MS: multiple sclerosis, HD: Huntington's disease, TBI: traumatic brain injury)

Table 1.

Key transcriptional factors for microglial development

Key Transcription Factors	Expression of Transcription Factor	Signaling Molecules	Role in Microglial Function	Effect on Microglial Population and Transcriptome	Ref
Interferon regulatory factor 8 (IRF8)	Highly expressed in the hematopoietic precursors	Heterodimeric partner of PU.1 and is a molecular target downstream of PU.1	Regulates early maturation of microglial precursor cells; regulates survival of microglia in early stage	IRF8-deficiency showed reduced number of mature precursor cells that are CD45+, CX3CR1HIGH, F4/80HIGH	[13, 18, 20, 77]
Myocyte enhancer factor 2a MEF2a	Specifically expressed in adult microglia	TGFB	Maintaining matured microglial phenotype and shapes epigenomic landscape	MEF2A motif analysis showed increased fraction of promoters associated with the regulator regions of adult microglia genes	[13, 34, 78]
Myocyte enhancer factor 2c MEF2C	Reduced in aged mice due to upregulation of type-1 interferon (IFN-α/β) expression	↑IFN1 > ↓MEF2C; TGFB	Aged microglia phenotype with increased secretion of pro-inflammatory cytokines; regulating neuronal synaptic plasticity	Injection of TNFa, a proinflammatory cytokine associated with aging, in MEF2C-deficient mice led to IBA1 intensity. Following LPS stimulation in vtiro, isolated from MEF2C-deficient microglia secreted more proinflammatory cytokines and reduced social preference behavior	[34, 79]
PU.1	Continuously expressed from EMPs to adulthood microglia	Downstream of RUNX1 during early development; TGFB	Maintains homeostatic genes and suppresses pro-inflammatory pathways	63% of microglial sensome genes are PU.1 targets: *Lst1*, *Hla-Dra*, *Cd11b*, *Mrc1*, *Cebpa*, *Trem2*, *Ptprc*, *Tyrobp*	[22]
Runt-related transcription factor 1 (RUNX1)	Highly expressed in the hematopoietic precursors	TGFB	Involved in proliferation and differentiation into ramified morphology	RUNX1-binding motif is enriched at the enhancer landscape of adult mouse and human microglia, suggesting a role in the maintenance of adult microglial phenotype	[22, 80-82]
Spalt like transcription factor 1 (SALL1)	Expressed during embryogenesis, upregulated in adult phase	TGFB	Maintains homeostatic genes and suppresses pro-inflammatory pathways	Increased homeostatic genes including *P2ry12*, *Serpinf1* or *Slc2a5*; reduced macrophage-related genes such as *Msr1*, *Cd69*, *Igf1* and *Spic* And reduced pro-inflammatory genes: *Axl*, *Nox2* and *Irf7*	[21, 24, 57, 83]
V-maf musculoapon eurotic fibrosarcoma oncogene homolog B (MAFB)	Specifically expressed in adult microglia, increased expression during aging	↑GM-CSF > ↓MAFB; TGFB	Role in adulthood microglia maturation and differentiation; suggested antagonistic relationship with the interferon pathway; “off-state factor” for regulating microglia response under stressed or pathological conditions, which regulates microglial gene signature, self-renewal and morphology	Promotes anti-inflammatory phenotypes and regulates expression of immune and viral genes; increased *Ctsh* and *Pmepa1* in the later adult stage indicating that Mafb suppresses antiviral response pathways; MAFB-deficient microglia show increased self-renewal and reduced *P2ry12* and *Ccl2* expression in the presence of GM-CSF treatment	[13, 84-88]

Table 2.

Epigenetic modification in microglia

Stimulation	Epigenetic Modification	Microglia Phenotype and Effect on Transcription Factor	Refs
Aβ accumulation and 1X or 4XLPS injection	H3K4me1 increased H3K4me1 levels in microglia from 1xLPS versus 4xLPS wildtype animals showed enrichment for the 'thyroid hormone signaling pathway', including a putative enhancer for hypoxia inducible factor-1α (HIF-1α). 4xLPS-treated APP animals showed increased H3K4me1 levels in putative enhancers related to phagocytic function.	Activation of HIF-1a and mTOR pathways leading to DAM or MGnD phenotype with enhanced phagocytosis	[11]
Aβ accumulation	Similar levels of H3K4me2 in AD microglia and DAM microglia	Suggested to be involved in priming of DAM phenotype since similar level of active H3K4me2 in AD microglia and DAM microglia	[41]
MECP2 deletion	Increased histone H4 acetylation at *cis*-regulatory regions of Fkbp5, a canonical glucocorticoid target gene, and recruitment of nuclear receptor co-repressor2 and histone deacetylase 3 (Hdac3) complex	Dysregulation of genes involved in glucocorticoid signaling, hypoxia response and inflammatory responses	[17]
Dying neurons	Clearance-specific genes display negligible amounts of H3K27me3 in cbMg, with higher cell death rate, as compared to in stMg. Dying cells *in vitro* increases the expression of H3K27me3-specific demethylases, which leads to reduced H3K27me3 activity.	Suppression of clearance genes and progressive induction of phagocytic genes	[42]

Table 3.

Epigenetic regulation of PU.1 expression in microglia

Model	Tissue treatment	Isolation method	PU.1 expression change	Dynamic changes	Ref
Maternal immune activation (MIA)	C57BL/6 5mg/kg PolyIC intraperitoneal injection at G15-Hippocampal tissue-> anti-inflammatory minocycline	Percoll gradient-> MACS beads with CD11b	↓PU.1	Downregulated genes included Fcgr1, Itgav, P2ry6, Sirpa, Siglece, Cx3cr1 that constitute the sensome in which microglia-neuron communicates	[59]
Early life stress	BALB/cByj mice separation procedure occurred daily from PND1-21; Hippocampus collected from P28 mice	Percoll gradient-> FACS CD11b, CD45	↓PU.1	reduced expression of pro-inflammatory genes such as Tlr3, IL17ra, increased expression of IL6, high rate of phagocytic activity, reduced expression of many developmental genes	[64]
Germ-free (GF)	6-20 weeks adult GF C57BL/6 mice, SPF mice treated with antibiotics (cefoxitin, gentamicin, metronidazole-> Recolonization of the gut with complex microbiota	density gradient-> FACS for CD11b and CD45	↑PU.1	Immature phenotype of GF microglia, reduced innate immune response, GF microglia failed to display activated morphology after LPS stimulation	[15]
Alzheimers Disease (AD)	CK-p25 3-month old female mice-hippocampus	IHC	↑PU.1	N/a	[89]
	PS19 tau transgenic mice-> C3aR KO mice	Myelin removal-> FACS with CD11b and CD45	↑PU.1	Increased volumes and reduced branching and complexity in microglia and astrocytes of PS19, which were corrected by C3aR-KO in mice.	[49]
	Human postmortem hippocampal; Human primary microglial-enriched culture-> Vorinostat (HDAC inhibitor)	IHC	↑PU.1	N/a	[30]
Huntingtons Disease (FID)	BV2 cell line; R6/2 mouse line with mHTT knock in Hdh175/175 mice -> siRNA – PU.1	Percoll gradient→ FACS with CD11b, CD45, CD16/32	Age/stage dependent ↑PU.1	Increased proinflammatory phenotype including IL6 expression	[23]
Traumatic Brain Injury (TBI)	adult male Spraque-Dawley rats lateral fluid percussion	IHC	↑PU.1	N/a	[51]

Table 4.

Maintenance of microglial gene signature in human iPSC-derived microglia-like cells

Cell type	Key Factors in Maintaining Microglia	Microglia Protein Expression	Method	Ref
Pluripotent stem cell-derived microglia-like cells (pMGLs)	IL34 and CSF1	CD45, IBA1, P2RY12, TMEM119	IHC, qPCR, and RNAseq showing pMGLs resemble primary fetal human and mouse microglia	[67]
Human microglial-like cells (iMGLs) differentiated from human iPSC	TGFβ, M-CSF, IL-34, insulin, CD200, CX3CL1	CD45, CX3CR1, TREM2, TGFβR1, P2RY12, MERTK, PROS1, and ITGB5	IHC, RNAseq PCA analysis showing close resemblance to human fetal and adult microglia	[68]
Human iPSC-derived microglia (iPSC-MG)	IL34 and GM-CSF	CD11b CD11c, CX3CR1, IBA1, P2RY12, TMEM119	IHC, Flow cytometry, and RNAseq revealing close similarities between iPSC-derived (iPSC-MG) and human primary microglia as well as clear distinctions from macrophages. mRNA levels of TMEM119 were very low in iPSC-MG compared with hMG, even though the protein was detected by immunofluorescence.	[69]
Human IPSC-derived Microglia co-culture them with iPSC-derived cortical neurons (co-pMG)	Astrocyte-derived GM-CSF, M-CSF, and TGFβ, IL-34	CD11b, CD14, CD45, IBA1, MERTK	IHC, Flow cytometry, qPCR, and RNAseq PCA showed weak neural cell signature in the co-cultured pMG isolated with CD11b beads, but close similarity of these cells to fetal microglia	[70]
Human and murine iPS-MG	Subsequent culture of iPS-HPC on astrocyte monolayers supplemented with GM-CSF, M-CSF and IL-3	CD11b and IBA1, HLA-DR, CD45, TREM-2 and CX3CR1	IHC, microarray hybridization and qPCR show human iPS-MG resembled those of human fetal microglia including expression of *P2RY12*, *GPR34*, *MERTK*, *C1QA*, *PROS1* and *GAS6* as well as those of DC and Mac human iPS-MG did not cluster as tightly with the commercially obtained human fetal microglia compared to murine analysis	[71]

Table 5.

Technical limitations in transcriptomic and epigenetic analysis of microglia

Limitations	Impact	Current trend	Future directions
Different microglia isolation methods	Difficulty in reproducing data and reaching consensus of microglial subpopulation function.	Identifying subpopulations using single-cell RNAseq	Provide standardized method using updated microglial markers.
RNAseq processing differences	The difference in reading depth can result in missing specific genes that are not highly expressed in microglia.		Provide optimal reading depth to analyze all genes possible.
	Different preparation methods of DNA libraries can lead to different reads of transcript due to fragmentation of mRNA.	SmartSeq, designed for small starting amounts, involves PCR amplification before the final fragmentation of the sequencing library TruSeq method using heat-fragmentation of mRNA and the only amplifies the sequencing library	Provide consensus of most efficient method for RNA preparation
Difference between protein expression and mRNA transcript level	Difference between transcriptional and protein level can lead to misinterpretation of gene expression changes.	Flow cytometry or IHC confirmation Mass spectrometry	Provide protein validation of identified genes.