



## INVITED REVIEW

## Epigenetic control of microglial immune responses

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## Summary

Microglia, the major population of brain-resident macrophages, are now recognized as a heterogeneous population comprising several cell subtypes with different (so far mostly supposed) functions in health and disease. A number of studies have performed molecular characterization of these different microglial activation states over the last years making use of “omics” technologies, that is transcriptomics, proteomics and, less frequently, epigenomics profiling. These approaches offer the possibility to identify disease mechanisms, discover novel diagnostic biomarkers, and develop new therapeutic strategies. Here, we focus on epigenetic profiling as a means to understand microglial immune responses beyond what other omics methods can offer, that is, revealing past and present molecular responses, gene regulatory networks and potential future response trajectories, and defining cell subtype-specific disease relevance through mapping non-coding genetic variants. We review the current knowledge in the field regarding epigenetic regulation of microglial identity and function, provide an exemplary analysis that demonstrates the advantages of performing joint transcriptomic and epigenomic profiling of single microglial cells and discuss how comprehensive epigenetic analyses may enhance our understanding of microglial pathophysiology.

## KEYWORDS

aging, Alzheimer, ATAC-seq, ChIP-seq, epigenetics, microglia

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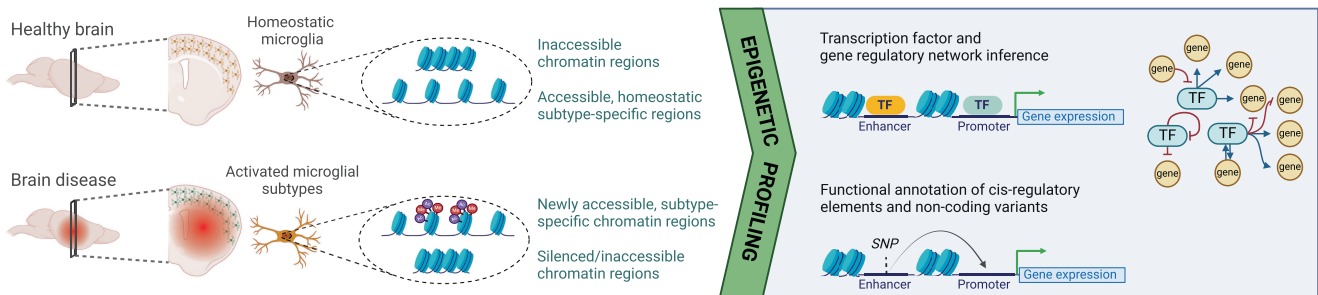
## 1 | WHY SHOULD WE STUDY EPIGENETIC ALTERATIONS TO UNDERSTAND MICROGLIAL PATHOPHYSIOLOGY?

Over the last ~5 years, a number of studies have analyzed the molecular responses of microglia in the context of development, aging, and brain disease using transcriptomics, whereas few studies have so far performed genome-wide analyses of microglial epigenomic profiles. While transcriptomics explores differences in gene expression levels at the time of cell/nucleus isolation and maps differentially expressed genes to their associated molecular pathways,<sup>1</sup> epigenomics examines modifications of the DNA of a cell that occur without changing the DNA sequence itself, including DNA methylation, histone modifications, chromatin remodeling as well as regulation by non-coding RNA expression (e.g., microRNAs).<sup>2,3</sup> These modifications, in turn, can alter gene expression and cellular function, and have been shown to be important for cell differentiation as well as cellular responses to aging and disease<sup>4-9</sup> (Figure 1A). Moreover, compared with transcriptomics, which provides a comprehensive snapshot of acutely expressed genes, epigenetic modifications can lead to both short- and long-term changes in gene expression and therefore microglial function (Figure 1B, and see below for details), and may (theoretically) even be passed on to the next generation of cells.<sup>10</sup>

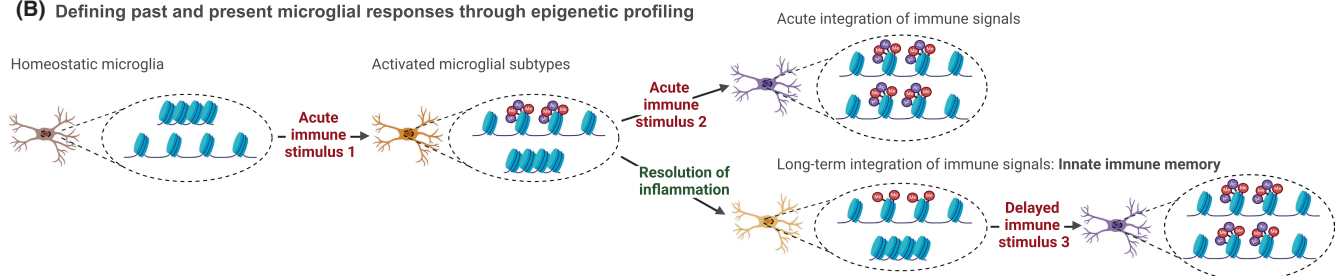
In the context of disease, it is worth noting that many disease-associated DNA sequence variants, as identified in genome-wide association studies (GWAS), are found in non-coding regions, that is, they do not modify the encoded protein itself but rather regulate its expression level in a particular cell type and in a particular cellular (or disease) context (Figure 1A). As these cis-regulatory elements (CREs), especially enhancer regions, can be far away from the genes they regulate, functional annotation is difficult and mainly lacking.<sup>2</sup> Understanding the impact of such disease-associated variants therefore will require comprehensive mapping of epigenetic modifications in CREs to determine in which cell subtype and under which specific circumstances (e.g., developmental or disease conditions) CREs regulate which target genes. Such complex analyses are now becoming possible through recent technological advances that allow epigenetic and transcriptomic profiling at single-cell level either in a parallel fashion (with post hoc computational mapping of epigenetic and transcriptomic alterations) or jointly from the very same cell. Such molecular maps will be highly informative with regard to cellular identity and function but are almost entirely lacking for microglial cells so far.

Probably the most studied and best understood epigenetic modifications in the context of macrophage biology are histone modifications. Histones are structural proteins that DNA can wrap around to form nucleosomes. When wound tightly around histones, DNA is relatively inaccessible, and transcription is therefore limited. In contrast,

### (A) Defining disease-associated microglial responses through epigenetic profiling



### (B) Defining past and present microglial responses through epigenetic profiling



**FIGURE 1** Epigenetic profiling can generate biological insight beyond other omics approaches. (A) Comparative analysis of microglial epigenetic states in the healthy versus diseased brain allows for construction of transcription factor (TF) and gene regulatory networks and enables mapping of disease-associated single nucleotide polymorphisms (SNPs) to microglial subtypes and their functional annotation. (B) Epigenetic profiles can also serve to reveal not only the current but also the past immune responses of microglia and inform on how immune signals can be integrated by microglia at the epigenetic level on the short (i.e., during acute inflammation) and long term. Long-term reprogramming is referred to as “innate immune memory,” where a first insult is followed by a refractory phase, which is characterized by persistent epigenetic modifications, and a subsequent stimulus then triggers a modified microglial response that results from prior epigenetic reprogramming in response to the initial stimulus.

open chromatin (i.e., regions where DNA is unwound from histones) generally reflects genomic regions that are accessible for binding of transcription factors and, in turn, gene expression.<sup>11</sup> Therefore, many studies use a method called *Assay for Transposase-Accessible chromatin using sequencing* (ATAC-seq) to assess chromatin accessibility and define genomic regions accessible for transcription.<sup>12</sup>

Because DNA accessibility and, in turn, gene expression ultimately define cell identity, chromatin accessibility is highly regulated and can be activated or suppressed through covalent modifications of DNA or its local histone proteins. These modifications depend on sequence-specific binding of transcription factors as well as chromatin-binding proteins, which become activated, for example, during development or in response to microenvironmental cues, leading to a cell identity- and cell environment-specific epigenetic profile. For example, a variety of covalent modifications can be added to histone tails, and how these regulate nucleosome structure depends on their position and type.<sup>13,14</sup> Histone marks therefore form a complex “epigenetic code” as both activating and repressive modifications may exist on histones of the same CREs, and their interplay defines DNA accessibility and thus gene transcription. For example, histone 3 acetylation at lysine 27 (H3K27ac) is a well-described example of an epigenetic modification that stimulates gene transcription. Accordingly, H3K27ac marks are enriched in accessible CREs, such as active promoters and enhancers.<sup>15,16</sup> At the same time, active enhancers also carry histone 3 lysine 4 monomethylation (H3K4me1), a modification that remains stable even if H3K27 tails become deacetylated, generating an epigenetic memory of previous molecular responses.<sup>17,18</sup> In addition to these permissive marks, repressive modifications, such as trimethylation of H3K27 (H3K27me3), can restrict chromatin accessibility. Because some histone modifications are relatively stable (at least for weeks to months), the epigenomic profile can reflect microenvironmental changes in the past of a cell that, in turn, can alter its future molecular responses—in the case of macrophages and microglia, such effects are referred to as innate immune memory (Figure 1B; and see below). Thus, the cellular epigenome provides a rich source of information with regards to a cell's past and present state that is arguably more informative than profiling gene expression alone.

## 2 | EPIGENETIC ORCHESTRATION OF MACROPHAGE TRANSCRIPTIONAL PROGRAMS

In the periphery, numerous studies over the last years have shed light on the epigenetic landscape of peripheral macrophages in much more detail than currently reported for microglia as the main CNS innate immune cell type. These advances were initially made possible by technological developments which allowed tracing, isolation, and analysis of macrophages at single-cell level in different tissues, enabling the molecular characterization of tissue-specific macrophage cell types and populations during development, under homeostatic condition but also in pathophysiological settings.<sup>19,20</sup> The initial observation that macrophage populations in many organs originate from the

same embryonic precursor cells, but develop distinct transcriptional programs, raised the question as to how tissue-specificity is encoded. Here, epigenetic profiling revealed that tissue-resident and recruited macrophages adapt to their microenvironment,<sup>16,21</sup> including insights into key transcription factors and epigenetic regulators that govern these molecular programs and ensure their context-dependent execution.

One key event in tissue-specific macrophage adaptation is the priming of the promoter and enhancer landscape to ensure that macrophage subtype-specific gene expression programs can be induced. This process is orchestrated by the combined activity of key lineage-determining transcription factors, such as PU.1, as well as signal-regulated activation of context-specific transcription factors, for instance of the C/EBP, AP-1, and RUNX families.<sup>22,23</sup> In concert, these highly microenvironment-specific transcription factors will bind to promoter and enhancer regions and enable a coordinated expression of a core macrophage as well as additional stimulus-dependent transcriptional modules<sup>24</sup> that together define macrophage identity and context-specific responses.

Over the last years, the epigenetic underpinnings enabling macrophage responses to pathological changes have been studied in detail for several peripheral macrophage populations. For instance, in a model of dietary non-alcoholic steatohepatitis, several thousand regions of open chromatin showed significant increases while a similar number of regions exhibited decreases in H3K27ac levels in the liver's macrophages, the Kupffer cells. Both sets of regions were linked to altered gene expression patterns.<sup>25</sup> This supports the notion that stimulus-specific alterations in the epigenetic landscape of macrophages are responsible for their highly diverse transcriptional responses to exogenous cues. Similar findings have been reported, for example, for macrophages in atherosclerotic plaques<sup>26,27</sup> or adipose tissue.<sup>28</sup>

In summary, there is a plethora of evidence suggesting that tissue-specific adaptation of macrophages, besides a core epigenetic and transcriptional differentiation program necessary for their identity, is governed by external cues in the tissue microenvironment that shape epigenetic and, in turn, transcriptional landscapes. Beyond these tissue-specific programs, macrophages can integrate many exogenous stimuli on both levels to fine-tune their specific response. Notably, microglia are the only tissue-resident macrophage population that derives entirely from early embryonic precursor cells in the yolk sac,<sup>29,30</sup> and other macrophages, even when transplanted into the brain, are unable to attain the precise molecular program of microglia.<sup>31</sup> This raised the question if microglia are programmed in a similar fashion, and we will discuss previous work on microglial epigenetic reprogramming in the following.

## 3 | ANALYSIS OF CHROMATIN ACCESSIBILITY ENHANCES UNDERSTANDING OF MICROGLIAL PHENOTYPES AND FUNCTION IN MOUSE MODELS AND HUMAN DISEASE STATES

As mentioned above, only a limited number of studies have so far investigated the epigenetic mechanisms controlling microglia

phenotypes in the healthy and diseased brain. However, the importance of analyzing the epigenetic control of microglial immune responses is highlighted for example by the fact that most sporadic Alzheimer's disease (AD) variants, discovered by large-scale GWAS studies, are located in microglia-specific enhancers. Therefore, a first publication by Nott et al. in 2019, which defined the human microglial enhancer repertoire, enabled an interpretation of these GWAS hits.<sup>15</sup> For instance, this study demonstrated that a microglia-specific enhancer for *BIN1* harbors the AD risk variant rs6733839, and validation experiments showed that deletion of this enhancer resulted in ablation of *BIN1* expression in induced pluripotent stem cell (iPS)-derived microglia, but not neurons or astrocytes, confirming its microglia-specific impact on AD pathogenesis.

Recent advances in high-throughput methods and multiomic data integration have prompted comprehensive studies looking at diverse microglial states in aging<sup>32</sup> and in AD.<sup>33–35</sup> For instance, through analyzing the transcriptional profiles of microglia in young (3 months), middle aged (14 months) and aged (24 months) wild-type mice, Li et al. uncovered a set of genes that are differentially regulated with aging, which the authors called age-dependent microglia (ADEM) genes.<sup>32</sup> Transcriptomic analyses were then complemented by profiling of open chromatin (using ATAC-seq), which revealed that ADEM genes also exhibited differential chromatin accessibility in their promoters. Epigenetic profiling also enabled the authors to study transcription factor (TF)-binding sites in differentially accessible chromatin regions. Fifty TFs with significant enrichment in ADEM-accessible regions were identified and their binding activities towards ADEM genes and total genes were analyzed. This revealed that CEBP $\beta$  (CCAAT enhancer binding protein- $\beta$ ) and MEF2C (myocyte enhancer factor 2C) showed enhanced and decreased binding activities in ADEM genes, respectively. Notably, this was despite the fact that both TFs showed an age-dependent increase of their binding activities in total genes, indicating a selective regulation of microglial gene expression by these TFs during aging that transcriptomic profiling alone would not have been able to reveal.

Interestingly, CEBP $\beta$  is associated with pro-inflammatory activation states<sup>36</sup> while MEF2C is a negative regulator of microglial pro-inflammatory responses,<sup>37</sup> in line with reports that microglia are primed during aging, that is, they show increased inflammatory responses compared to microglia in adult animals.<sup>38</sup> However, while aged microglia showed enhanced expression of inflammation-related genes (such as the proinflammatory cytokine IL-1 $\beta$  as well as genes involved in lipid metabolism, incl. *Apoe*), they did not show stronger gene expression changes in response to a second stimulus, namely peripheral challenge with bacterial lipopolysaccharides (LPS) in the study by Li et al.<sup>32</sup> In addition, ATAC-seq revealed that aging and LPS challenge both induced significant but largely distinct chromatin changes in microglia, with chromatin accessibility altered by aging being less prominent compared to the changes induced by LPS challenge,<sup>32</sup> in line with an acute and pronounced immune activation in response to LPS and

a chronic low-grade inflammatory response being triggered by the aging process in the brain.

Recent studies have also started to investigate epigenetic alterations in microglia in post-mortem human tissue. For instance, through integrating data from single-cell RNA-seq and ATAC-seq experiments performed on nuclei isolated from human post-mortem brain tissue, a recent study by Sun et al. uncovered diverse states of microglia at different stages of AD pathology.<sup>33</sup> In this work, transcriptome analysis identified 12 microglial clusters, including a homeostatic state (MG0), three inflammatory states (MG2, MG8 and MG10), a lipid processing state (MG4), and a glycolytic state (MG7).<sup>33</sup> In comparison, transcriptomic signatures of so-called disease-associated microglia (DAM) were previously reported in mouse models of A $\beta$  pathology,<sup>20,39,40</sup> which separated as distinct populations in single-cell clustering analyses. Mapping these signatures onto their dataset, however, Sun et al. found them to be distributed across multiple subtypes of human microglia, including the ribosome biogenesis-related state MG3, the lipid processing state MG4 and one of the inflammatory states, MG10, indicating that (maybe not surprisingly) the human microglial response to fully manifested AD pathology (incl. a variety of pathological and age-related changes) is more complex than observed in murine models of A $\beta$  pathology and still remains to be resolved with regards to the role of different microglial subtypes in AD pathogenesis.

Due to the dynamic nature of the microglial immune responses, the authors asked whether the three inflammatory states (MG2, MG8, MG10) could be part of a trajectory rather than individual activation states. To test this hypothesis, multiplexed in situ hybridization was performed for a panel of signature genes representing the three inflammatory microglial subtypes. This analysis revealed that subtype markers often overlapped, suggesting that the three inflammatory states of microglia likely reflect transitions that occur with progression of their inflammatory response.

Moreover, focused analysis of TFs revealed several candidates that were enriched in specific microglial states. For instance, *HIF1A* was prominently expressed and found to be regulating the marker genes in the glycolytic state MG7, as well as the stress-related state MG6 and one of the inflammatory states, MG10. This is in line with the reported function of HIF-1 $\alpha$  as a master regulator of the metabolic switch from oxidative phosphorylation to glycolysis, as well as microglial stress and inflammatory responses.<sup>41–44</sup> Interestingly, HIF-1 $\alpha$  was also among three TFs that were found to regulate the transition from the weak inflammatory state MG8 to strong inflammatory state MG10, as predicted by a screening experiment in iPS-derived microglia-like cells (iMGLs). In particular, snRNA-seq on CRISPR-edited iMGLs stimulated with LPS showed that ablation of three TFs (*FOXO3*, *FOXP2* and *HIF1A*) was sufficient to prevent the induction of a MG10 state marker, IL-1 $\beta$ , which induced the transition to a strong inflammatory state in control iMGLs. Importantly, although activation states of iMGLs transcriptomically resembled in vivo microglia states, the homeostatic state MG0 observed in microglia isolated from post-mortem tissue was not fully represented in iMGLs,<sup>33</sup> highlighting the importance of the tissue environment



for specific gene expression programs, as demonstrated previously in mouse models.<sup>31</sup>

Notably, the authors also analyzed the epigenetic state of human microglia through performing snATAC-seq on post-mortem brain tissue from a subcohort of the same individuals used for their transcriptomic analysis. Surprisingly, this revealed only three distinct epigenetic subtypes (as opposed to 12 transcriptomic subtypes), comprising a homeostatic state and two activated states. This indicates that the rich diversity of microglial transcriptional states might not be fully represented by their epigenetic states, when assessed based on their chromatin accessibility profiles. This potential limitation of scATAC-seq has also been reported for Kupffer cells [Ref. 25 and reviewed in 45]. Nevertheless, the peaks in activated microglial states were significantly enriched in genes related to a cellular interleukin response, regulation of blood brain barrier formation and integrity, as well as cytotoxic T cell differentiation, indicating that additional biological information can be derived from analysis of the microglial epigenome, although this was not further validated.

Nevertheless, through combining their transcriptomic and epigenomic datasets, the authors were able to build a co-expression network for AD-risk genes as identified by genome- or transcriptome-wide association study (GWAS and TWAS, respectively) and could infer the upstream TFs of differentially expressed genes (DEGs). Interestingly, among the seven regulators that showed significant associations in the category GWAS/TWAS, GWAS/TF or TWAS/TF, *BIN1* and *RELB* were highly correlated and differentially expressed in microglial subtypes. This is in line with their reported roles in regulating neuroinflammation.<sup>46–48</sup>

In contrast to the work by Sun et al., which suggested that snATAC-seq profiles provide less cellular subtype resolution compared with snRNA-seq profiles,<sup>33</sup> a prior study on human post-mortem brain tissue of AD patients and cognitively healthy controls by Morabito et al. identified three microglia clusters based on snRNA-seq but five microglia clusters from (parallel analysis of) snATAC-seq.<sup>34</sup> By integrating the two datasets, the authors discovered that the proportions of two of the epigenetically-defined microglial clusters were increased in late-stage AD. These two clusters were both mapped to the activated microglia cluster MG1 identified by snRNA-seq, which was also increased in AD compared to control patients.<sup>34</sup> Interestingly, the motif variability of the microglial TF SPI1 (also known as PU.1) in the snATAC-seq data was only increased in these two clusters, with its target genes downregulated in MG1. This indicated a role of SPI1 as a transcriptional repressor in late-stage AD. The authors then compared their human signatures to the mouse DAM phenotypes, which have been reported to transition through an early (DAM 1) to a late (DAM 2) stage.<sup>20</sup> Notably, through trajectory inference the authors found that with disease progression, homeostatic signatures were decreased, while stage 1 DAM signatures were increased; however, contrary to mouse models of A $\beta$  pathology, they observed a global depletion of the stage 2 DAM signatures. Further analysis of the integrated microglia trajectory constructed with nuclei from both snATAC-seq and snRNA-seq datasets revealed

that the trajectory for SPI1 motif variability was negatively correlated with gene expression at advanced disease stages, confirming its role as a transcriptional repressor.<sup>34</sup> Interestingly, a mouse study in APP/PS1 mice showed that the binding landscape of SPI1 can be enhanced by IL-33 administration,<sup>49</sup> where IL-33 injection in mice induced a transcriptionally distinct microglia subpopulation, characterized by upregulated major histocompatibility complex class II (MHC-II) genes, which promoted A $\beta$  clearance. In contrast, independent studies have demonstrated that reduced SPI1 gene dosage induces a protective microglial phenotype in mouse models and AD patients.<sup>50,51</sup> Nevertheless, these data indicate that SPI1/PU.1 regulates a pathologically relevant microglial response in AD. In contrast, DAM cells (as reported in mouse models) may not present as a distinct population in the human brain.

#### 4 | HISTONE MODIFICATIONS MODULATE MICROGLIAL PHENOTYPES IN MOUSE MODELS OF ALZHEIMER'S DISEASE

Several studies in mouse models have also demonstrated regulation of histone modifications in shaping microglia phenotypes in the context of development, homeostasis, and AD. For instance, prenatal microglia-specific deletion of histone deacetylase-1 and -2 (*Hdac1/2*) resulted in enhanced H3K9 and H3K27 acetylation at the proximal promoters of genes regulating cell cycle and cell activation, which led to impaired microglial development.<sup>52</sup> Interestingly, *Hdac1* and *Hdac2* were dispensable for maintenance of microglia in adult mice; however, knocking out both genes in microglia of *5xFAD* mice, a mouse model for A $\beta$  pathology, increased amyloid phagocytosis in microglia and ameliorated A $\beta$  burden. Another study in the same mouse model profiled H4K12 lactylation (H4K12la) and detected increased H4K12la levels in *5xFAD* mice and also in AD patients, particularly in microglia adjacent to amyloid plaques.<sup>53</sup> Notably, plaque-associated microglia have been shown to upregulate glycolytic genes such as *Hif1a*, *Pkm2*, and *Lhda*, suggesting a glycolysis/H4K12la/PKM2-positive feedback loop in microglia responding to A $\beta$  pathology. Indeed, interference with this feedback loop through microglia-specific deletion of *Pkm2* in *5xFAD* mice led to reduced levels of H4K12 lactylation, attenuated neuroinflammation, ameliorated A $\beta$  burden and improved cognitive function in this mouse model for AD pathology. Thus, this study demonstrated a detrimental link between glycolytic metabolism, epigenetic reprogramming and microglial function that may serve as a novel immunomodulatory target in AD.

#### 5 | REGULATION OF MICROGLIAL EPIGENETIC REPROGRAMMING BY THE MICROBIOME

In a previous study on germ-free (GF) mice, we also reported that microbiomes can differentially regulate gene expression and

chromatin accessibility of microglia in male and female mice during development and adulthood.<sup>54</sup> When compared to specific-pathogen-free (SPF) mice, the lack of a microbiome in GF mice resulted in less chromatin accessibility in embryonic microglia and slightly altered chromatin landscape in adult microglia, reflected by changes in their differentially accessible chromatin regions (DARs) and transcriptomic profiles, leading, for example, to altered microglial colonization of the cortex. A recent study by Erny et al. further explored the effect of the absence of microbiome on microglial functions.<sup>55</sup> Chromatin immunoprecipitation with sequencing (ChIP-seq) of histone 3 lysine 4 trimethylation (H3K4me3) and histone 3 lysine 9 acetylation (H3K9ac) from isolated microglia from GF and SPF mice revealed higher abundance of H3K4me3 and H3K9ac at the promoters of metabolic genes in GF microglia compared with SPF microglia, leading to altered microglial metabolic states in GF mice.<sup>55</sup> Interestingly, treatment with acetate, a microbiome-derived short-chain fatty acid, restored the metabolic fitness in steady state microglia and partially reversed the effect of GF condition on microglial phenotype and A $\beta$  deposition in 5xFAD mice. However, the authors did not investigate whether histone methylation and acetylation marks were also altered by this treatment.

## 6 | BRAIN REGION-SPECIFIC MICROGLIAL FUNCTIONS CAN BE REGULATED THROUGH EPIGENETIC MECHANISMS

Previous studies have indicated that microglial phenotypes and functions differ according to brain regions,<sup>56–58</sup> but only one study has so far directly investigated the role of epigenetic mechanisms in controlling these region-specific adaptations. In particular, microglia in the cerebellum are more phagocytically active than in the cortex and striatum due probably to a higher turnover of cerebellar neurons. Ayata et al. found that this brain region-specific clearance activity of microglia was regulated epigenetically, involving the polycomb repressive complex 2 (PRC2) which deposits the repressive histone modification H3K27me3.<sup>59</sup> The phenotype of cerebellar microglia was found to be due to higher expression of genes encoding for demethylases that mediate the removal of H3K27me3, with striatal and cortical microglia showing a substantial enrichment of H3K27me3 at the transcriptional start sites of phagocytic genes, and accordingly, lower clearance activity. Correspondingly, genetic inactivation of core components of PRC2 in striatal microglia led to upregulation of phagocytic genes and TFs enriched in cerebellar microglia, also increasing lysosomal content.<sup>59</sup> This transition in microglia from a surveillance phenotype towards a clearance-promoting state resembles the observations in neurodegenerative diseases,<sup>60,61</sup> where microglia may be phagocytically challenged either due to neurodegeneration or aggregated proteins.

## 7 | LONG-TERM EPIGENETIC REPROGRAMMING OF PERIPHERAL MACROPHAGES: INNATE IMMUNE MEMORY OUTSIDE OF THE BRAIN

The above studies clearly indicate that during acute inflammation or microenvironmental changes, exogenous signals drive epigenetic and, in turn, transcriptional and functional changes in tissue-resident macrophages, including microglia. Based on these observations, the question arose why such epigenetic changes occur and whether they may support specific functions of these cells. Netea and colleagues proposed that primary immune challenges can enhance responsiveness to subsequent triggers in myeloid cells, a phenomenon termed “trained immunity,” which effectively encodes innate immune memory.<sup>62</sup> Initial evidence for this concept derived from reports that certain infections and vaccinations can induce broad protection against later infection with other pathogens through innate immune mechanisms.<sup>63–65</sup> This was further supported by observations that bacterial lipopolysaccharides (LPS) and other Toll-like receptor ligands can induce a diminished inflammatory response upon subsequent stimulation, an observation described initially as *LPS tolerance*.<sup>66</sup> Such macrophage reprogramming was found to be mediated through the detection of pathogens and tissue damage by cells of the innate immune system, accomplished through pattern recognition receptors (PRRs) that are encoded in the germline.<sup>67</sup> These receptors are designed to recognize various molecular patterns indicative of damage or danger to the host, resulting from tissue injury or pathogen invasion, respectively. In addition to the immediate responses of macrophages to activation of PRRs, it was reported that their activation in animals via various microbial ligands can confer protection against subsequent lethal infections in a non-specific manner. For instance, challenge with a fungal ligand,  $\beta$ -glucan, provides protection against subsequent *Staphylococcus aureus* infections,<sup>68,69</sup> while the bacterial peptidoglycan component, muramyl dipeptide, confers protection against *Streptococcus pneumoniae* and *Toxoplasma gondii* infections.<sup>70</sup> Similarly, priming via administration of microbial mimetics, CpG oligodeoxynucleotides, can result in protection against experimental sepsis and meningitis,<sup>71</sup> while treatment with bacterial flagellins induces protection against *Streptococcus pneumoniae* and rotavirus.<sup>72,73</sup> Taken together, the current evidence supports a model where infections or exposure to immune stimulatory agents derived from microorganisms can trigger not only specific protection against reinfection with the same pathogen but also non-specific protection against subsequent challenges with distinct pathogens.<sup>74–76</sup> On a cellular level this protection is mediated by myeloid cells and, in particular, monocytes and tissue-resident macrophages.<sup>75–77</sup>

But how is this innate immune memory encoded on the molecular level? With the primary observation being that in myeloid cells enhanced, expedited, or qualitatively distinct transcriptional responses were elicited upon exposure to subsequent stimuli, the idea quickly arose that memory would have to be encoded in the

regulatory layers orchestrating gene expression. Indeed, factors like transcription of long non-coding RNAs (lncRNAs), DNA methylation,<sup>78</sup> reprogramming of cellular metabolism, and chromatin organization at the level of the topologically associated domains (TADs) have all now been connected to innate immune training.<sup>79</sup>

Numerous studies over the last years have now illustrated that activation of innate immune cells induces an epigenetic imprint that alters the long-term responsiveness of these cells and gives rise to functional programs characteristic for trained immunity. Central to the presence of such epigenetic imprints are two key epigenetic modifications: the acquisition of H3K27ac at distal enhancers (which are also marked by H3K4me1) and the establishment of trimethylation of H3K4 at the promoters of activated genes.<sup>17,64,66,80</sup> Together with altered 3D configuration of TADs this will ultimately result in altered chromatin accessibility, allowing for easier transcription once a secondary stimulus is encountered by a primed cell. If and how this information can be inherited throughout cell division remains one of the important questions to be resolved.<sup>81</sup>

## 8 | LONG-TERM EPIGENETIC MODULATION OF MICROGLIAL IMMUNE RESPONSES FOLLOWING PERIPHERAL INFLAMMATION MANIFESTS AS INNATE IMMUNE MEMORY

Similar to the initial observations in peripheral macrophages, several publications over the years indicated that inflammatory insults could have a long-lasting impact on the brain's immune response (see e.g., Refs. [82–85]); however, the first evidence that microglia can develop and retain an epigenetic memory of inflammatory events only emerged relatively recently. A first study by Schaafsma et al. demonstrated that administration of a single peripheral (i.e., intraperitoneal) dose of bacterial lipopolysaccharides (LPS; 1 mg/kg) resulted in suppression of cytokine expression and release when animals received a second dose of LPS four weeks later.<sup>86</sup> The authors demonstrated that this immune tolerance effect was mediated at least partially through reduced levels of H3K4me3 and increased levels of repressive H3K9me2 (but unaltered H3K27me3) in the promoters of IL-1 $\beta$  and TNF- $\alpha$ . Notably, this tolerant state did not only reduce pro-inflammatory responses but also led to enhanced microglial phagocytic activity upon re-stimulation.<sup>86</sup> A follow-up study by Zhang et al. provided further details regarding the mechanisms of innate immune memory in microglia through analyzing not only epigenetic reprogramming resulting from LPS injection but also in a model of accelerated aging due to genotoxic stress, the *Ercc1*-deficient mouse line.<sup>87</sup> In this study, the authors first characterized the transcriptional activation state of isolated microglia after stimulation with LPS (again at 1 mg/kg) as the second trigger and found that the primary insult, that is, LPS versus genotoxic stress, significantly altered microglial responses. In particular, a first dose of LPS caused suppressed immune responses, reflecting the group's previous work,<sup>86</sup> while genotoxic stress enhanced pro-inflammatory gene expression.

Notably, LPS-induced tolerance was selective for a subset of genes involved in pro-inflammatory responses (e.g., “positive regulation of immune response”), while other pathways involved in antimicrobial effector functions were less or not at all affected. In contrast, microglia primed by genotoxic stress in *Ercc1*-deficient animals showed significantly enhanced expression of many genes related to pathways associated with “innate immunity” and “inflammatory response.” Of particular interest for the concept of innate immune memory in microglia, the transcriptomic profiles of microglia from control animals and from animals four weeks after LPS treatment were almost indistinguishable, and modulation of transcriptional responses only became apparent upon restimulation. Similarly, a number of genes whose expression was unaltered in wildtype versus *Ercc1*-deficient animals without LPS treatment showed increased expression after LPS stimulation in *Ercc1*-deficient versus wildtype mice. In line with the authors' previous work, these findings indicated that an additional layer of molecular regulation must be present, likely at the epigenetic level, and only becomes apparent when the cells mount their next immune response.

To examine the epigenetic regulation of the observed microglial responses in detail, Zhang and colleagues performed analysis of open chromatin regions (ATAC-seq) as well as ChIP-seq for several histone modifications, namely H3K4me3 to identify active promoters and H3K27ac to identify active enhancers, as well as the repressive H3K27me3 mark. With regards to LPS stimulation (as the first or second stimulus), gene expression changes correlated with open chromatin regions as well as H3K4me3 and H3K27Ac enrichment in gene-associated promoters and enhancers. In contrast, the tolerance effect seemed to be mediated selectively by changes in enhancer regions, as repressed genes showed reduced ATAC and H3K27ac peaks only in enhancers but not promoter regions, and accordingly no difference in H3K4me3 levels in promoter regions were observed in tolerized microglia. Similarly, gene expression in *Ercc1*-deficient animals largely correlated with chromatin accessibility and enrichment of H3K4me3 and H3K27ac in promoters and enhancers, respectively, as well as showing depletion of the repressive H3K27me3 mark; this indicated that the ongoing inflammatory response in this model leads to an epigenetic state that is largely permissive for enhanced gene expression upon inflammatory stimulation. Of note, the chromatin state of microglia during an ongoing (and in the *Ercc1*-deficient mice also chronic) inflammatory response may differ from a context where a temporally-defined stimulus has subsided (as we previously discussed<sup>88</sup>), and where microglial gene expression profiles have returned to an apparently homeostatic state. However, such direct comparisons have so far not been performed and require further analysis.

Following the first indication of epigenetic control of microglial immune responses by Schaafsma et al.,<sup>86</sup> we subsequently demonstrated that microglia can develop not only immune tolerance but also immune training, which lasts for many months in mice and is sufficient to modulate brain pathology.<sup>18</sup> In particular, we found that a single peripheral injection of LPS (at a lower dose of 0.5 mg/kg compared to Schaafsma et al.<sup>86</sup>) induced acute immune

training effects, as evidenced by significantly higher levels of pro-inflammatory cytokines (e.g., TNF- $\alpha$ , IL-1 $\beta$ ) being released upon injection of a second peripheral LPS dose one day later. Notably, this immune training effect in the brain was uncoupled from the periphery, where immune tolerance developed already after the first LPS injection. With further peripheral doses of LPS, immune tolerance also developed in the brain, with IL-1 $\beta$  and TNF- $\alpha$  and other pro-inflammatory cytokines declining sharply, while the immuno-modulatory cytokine IL-10 remained elevated; this indicated that the modulation of immune responses was specific for certain inflammatory signaling pathways. To gain a first indication of whether these effects were driven by microglia and were mediated through epigenetic reprogramming, we genetically deleted histone deacetylase-1 and -2 (*Hdac1/2*) in microglia using inducible Cre expression under the microglial *Cx3cr1* promoter. While *Hdac1/2* deletion did not affect the initial cytokine release in response to the first LPS injection, the second dose no longer triggered immune training, indicating that immune memory in the brain (in response to peripheral LPS treatment) is predominantly driven by microglial cells and likely involves epigenetic mechanisms.

Based on these initial observations, we wanted to examine whether such acute peripheral insults could have long-lasting effects on microglial immune responses and might thereby modulate the pathogenesis of neurological diseases. If so, such mechanisms might explain how peripheral inflammation can alter the risk for developing neurological disease in patients, as recognized in epidemiological studies.<sup>89–92</sup> To test this hypothesis, we treated 3-month-old APP23 transgenic animals (which develop A $\beta$  plaque pathology starting from 6 months of age<sup>93</sup>) and wildtype control animals with either one or four peripheral LPS doses and examined pathology as well as microglial responses at the epigenetic, transcriptional, and functional level six months later.<sup>18</sup> Based on previous publications investigating innate immune memory in peripheral macrophages, we decided to focus on the microglial enhancer repertoire, using H3K4me1 and H3K27ac marks to detect poised (H3K4me1 only) and active (H3K4me1 and H3K27ac) enhancers (please note: due to the lack of comprehensive enhancer-gene linkage maps in mouse microglia, all enhancers described in this and other studies can only be considered putative, and their associated genes are inferred based on proximity, which is not necessarily correct or complete). We predicted that differences in poised enhancers (i.e., showing higher levels of H3K4me1 marks) would be evident in microglia from all animals receiving a single or repeated LPS injections, independent of genotype. Indeed, using ChIP-seq, we found that the poised enhancer repertoire was changed both in wild-type and APP transgenic animals 6 months after the initial immune insult and differed between a single and four LPS injections, in line with the training versus tolerance effects observed acutely after LPS injections, and indicating that long-lasting epigenetic reprogramming of microglia had occurred. Next, we examined the active enhancer repertoire, predicting that it would differ only in animals with a second inflammatory insult. In our study, this second insult occurred in APP transgenic animals, where

microglia mount an inflammatory response to A $\beta$  plaque pathology. In line with this prediction and reflecting the concept of innate immune memory, very few active enhancers were altered in microglia isolated from wildtype animals (despite significant changes at the level of poised enhancers, see above). In contrast, in microglia from APP transgenic animals, we observed differentially regulated and distinct active enhancer regions resulting from a single or four LPS injections when compared to vehicle-treated animals. Strikingly, the most pronounced differences were evident when comparing trained microglia from APP23 animals with a single LPS injection versus tolerized microglia from APP23 animals with four LPS injections: Here, a large number of enhancers were activated (i.e., showed increased levels of H3K27ac) in trained microglia but none in tolerized cells, corroborating the dichotomy between immune training and tolerance also in microglia. Our findings that tolerized microglia showed a substantial increase in poised but not active enhancers in APP transgenic animals indicated that repressive mechanisms likely provide an additional layer of epigenetic regulation as highlighted also by the publications from Schaafsma and Zhang et al.<sup>86,87</sup> Such mechanisms warrant further attention.

Given the pronounced differences in the microglial active enhancer repertoire in APP23 animals with one versus four LPS injections, we wanted to understand their impact on microglial gene expression and functional phenotypes. To this end, we performed bulk RNA-seq of microglia isolated from matching experimental groups. Reflecting the lack of enhancer-gene interaction maps, we found only a modest (albeit significant) concordance of 58% between differentially activated (putative) enhancers and expression of their nearest genes. However, when we performed weighted gene-correlation network analysis (WGCNA), several modules were identified that reflected alterations observed in active enhancers at the pathway level. In particular, in trained microglia from APP animals that received a single LPS dose, the top pathway regulated at the active enhancer level was the *Hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) signaling pathway*, which was also one of the top hits in the most significantly upregulated WGCNA module. Similarly, in tolerized microglia from APP animals treated with four LPS doses, the only significant pathway at the active enhancer level was the *Rap1 signaling pathway*, which again was part of a WGCNA module that we found to be downregulated in microglia from vehicle- and single LPS-treated APP animals but not in microglia from repeatedly LPS-treated animals compared to wildtype controls, indicating that immune tolerance inhibited this reduction in Rap1 signaling. Functional analyses confirmed these findings, with activation of HIF-1 $\alpha$  in trained microglia resulting in hyperpolarized mitochondrial membrane potential (as described in peripheral macrophages<sup>94</sup>) while tolerized microglia showed enhanced phagocytic activity, reflecting the reported involvement of Rap1 signaling in phagocytosis<sup>95</sup> and corroborating the findings by Schaafsma et al.<sup>86</sup>

Thus, our data indicated that peripheral immune stimulation could differentially affect transcriptional profiles and the function of microglia through epigenetic reprogramming. We therefore investigated if these changes were sufficient to modulate much

later developing brain pathology. Notably, we found that trained microglia promoted while tolerized microglia reduced A $\beta$  pathology by about 30% compared to vehicle-treated animals. These results are in line with the idea that peripheral immune insults may have long-lasting consequences on brain immunity and the progression of neurological disease. Therefore, these findings do not only provide a possible mechanism as to how peripheral inflammation may modulate risk for neurodegenerative diseases<sup>96,97</sup> but are also highly relevant in the context of viral pandemics, where the long-term consequences of infections on the brain are barely understood, but where neurological sequelae are common.<sup>98</sup> Of note, the effects of peripheral inflammation are not limited to microglial cells but are also evident transcriptionally in vascular as well as other glial cell types and neurons, with specific effects on pathology-associated cell types, for example, on microglia that surround A $\beta$  plaques.<sup>99</sup> The interplay and long-term effects of such multiple insults on different brain cell types, however, remain unexplored. In this context, epigenetic analyses could provide further insights into the mechanisms of signal integration and may reveal which cell types are capable of long-term epigenetic reprogramming and “immune memory” or may be rendered more vulnerable to secondary insults.

## 9 | TECHNOLOGIES FOR MULTIMODAL SINGLE CELL ANALYSES

One potential avenue to further assess immune responses and memory is the combined analysis of single-cell transcriptomes in combination with additional modalities. In recent years, there have been significant advances in methods that can profile the epigenome and transcriptome of the same cell. Initially, these methods relied on physically separating RNA from DNA or the nucleus and profiling these separated fractions independently. However, these methods were laborious and only allowed for low throughput. Lately, technologies have been developed that use differential labeling of RNA and epigenetic information encoded in DNA with subsequent separation of reads originating from both through restriction digestion, PCR, or molecular barcoding. Unlike the earlier methods, these newer methods do not require upfront separation and are therefore more readily parallelized in higher throughput.

Most commonly used, and the main focus of our work, are methods that jointly profile the transcriptome and chromatin accessibility. Here, accessible DNA is recovered as transposon-insertion-flanked regions using an assay for transposase-accessible chromatin (ATAC). The main breakthroughs in this context have been achieved by methods which use either combinatorial indexing, like SHARE-seq,<sup>100</sup> SNARE-seq2,<sup>101</sup> Paired-seq<sup>102</sup> or sci-CAR-seq<sup>103</sup> or the usage of microfluidics for cell or nuclei barcoding including the commercially available 10x Genomics Multiome technology, ASTAR-seq<sup>104</sup> and ISSAAC-seq,<sup>105</sup> while plate-based methods, for example, Smart3-ATAC<sup>106</sup> or scCAT-seq<sup>107</sup> are targeted only to specific research questions because of their much lower throughput.

When selecting a tagmentation-based method, it is important to consider the ease of use of commercially available methods, the higher throughput of combinatorial indexing-based methods, and the typically lower cost of non-commercial, laboratory-developed methods. However, non-commercial methods may be more difficult to establish in the laboratory. It is also important to consider the coverage, sensitivity, and specificity obtained across cellular modalities; unfortunately, systematic benchmarking of these methods is currently lacking. In most methods, transcriptome and accessible chromatin libraries are prepared in a common reaction and separated after indexing using magnetic beads, restriction enzymes, or specific PCR primers. Joint profiling of transcriptome and chromatin accessibility has a key advantage in that the link between gene expression and TF binding is more readily available and can be compared directly.

Advantages of multiomic analyses in dynamic systems are the possibility to directly assign TF activity to target genes. Moreover, differentiation trajectories can be inferred, and TF activity can be predicted before the TFs are expressed. Their activity is revealed in ATAC-seq profiles in pseudotime as binding sites become accessible before expression of the associated target genes. The ease of use of some of these methods has spearheaded their application in biomedicine and other domains; however, their application to microglia and myeloid cells in the CNS has to our knowledge not been reported, despite the clear gap in knowledge that exists.

One additional layer to the quantification of open chromatin would be the possibility to address the causes of accessibility changes. As outlined above, these are often the consequence of post-translational modifications of histone proteins. Histone modifications at single-cell resolution are informative in understanding epigenetic programs and differentiation trajectories of cells, thereby aiding in cell-state prediction. In addition to the well-established ChIP-seq<sup>108</sup> and cleavage under targets and release using nuclease (CUT&RUN) methods for profiling histone modifications in cell populations,<sup>109</sup> cleavage under targets and tagmentation (CUT&Tag) uses Tn5 transposase to directly tagment the antibody binding site,<sup>110</sup> enabling profiling of histone modifications at the single-cell level. Methods such as scCUT&Tag<sup>111</sup> and scSET-seq<sup>112</sup> use tagmentation directed by antibodies to profile active and silenced regulatory elements genome-wide by targeting domains bound by RNA polymerase II and Polycomb repressive complexes. Distinct variations on these methods which involve two epitopes being targeted using different antibody-conjugated transposases are scCUT&Tag-2for1<sup>113</sup> and scMulti-CUT&Tag<sup>114</sup> which can be used to assess the distribution of both epitopes in a single cell.

Extensions to these assays are multiple target identification by tagmentation (Multi-Tag), which can profile multiple chromatin features simultaneously in single cells using an antibody barcoding approach allowing for detection of up to three histone modifications in the same cell<sup>115</sup> and single-cell chromatin immuno-cleavage and unmixing sequencing (scChIX-seq), which maps two histone marks together in single cells, then computationally deconvolves the signal using training data from respective histone mark profiles.<sup>116</sup>



These methods focusing on the analysis of multiple histone modifications in single cells have been extended to characterize RNA and histone modifications using multi-omics techniques such as Paired-Tag<sup>117</sup> and coTECH<sup>118</sup> which use combinatorial barcoding to allow high-throughput detection of both transcriptome and chromatin occupancy. Additionally, multimodal assays for profiling protein–DNA interactions and the abundance of surface proteins in single cells have been developed.<sup>119</sup>

Taken together, based on these exciting developments, we expect it to only be a matter of time until a better understanding will be obtained with regards to how mRNA expression, chromatin accessibility and changes in histone modifications are shaping microglia properties at the single-cell level, and how they are affecting microglial phenotypes in homeostatic and inflammatory conditions.

## 10 | MULTIMODAL ANALYSIS OF MICROGLIA AT SINGLE-CELL LEVEL

As outlined above, analyzing the epigenetic profiles of microglia can yield novel insights into their responses to pathological insults and how these are orchestrated at the molecular level, but few studies have so far endeavored to perform comprehensive analyses of microglial epigenetic states and their impact on transcriptomic and functional responses of microglia. To perform joint epigenomic/transcriptomic profiling at single-cell level and at large scale, we recently optimized the SHARE-seq protocol, which allows for joint RNA- and ATAC-sequencing of single nuclei,<sup>100</sup> for analysis of microglial nuclei from fresh frozen brain tissue.<sup>120</sup> In a *proof-of-principle* pilot experiment, we then performed SHARE-seq of four fresh frozen brains (i.e.,  $n = 1/\text{condition}$ ) from 3- and 20-month-old wildtype and 12- and 20-month-old APP23 animals (which show A $\beta$  plaque deposition starting from 6 months of age). After quality control, we retained 9327 microglial nuclei for analysis (Figures 2A–C). We then asked whether the inclusion of epigenetic data would impact microglial subtype classification. We therefore first performed clustering analysis based on snRNA-seq and snATAC-seq data alone or using the multiome profiles. Based on snRNA-seq data, we identified nine distinct microglial subtypes (clusters R0–R8), while snATAC-seq data distinguished only six clusters (A0–A5), reflecting findings from previous parallel snRNA-seq and snATAC-seq analyses<sup>33</sup> and indicating that open chromatin regions may be insufficient to fully capture microglial heterogeneity. However, taking both data sets into account led to further resolution of microglial subtypes as evident by identification of 10 clusters (M0–M9) in

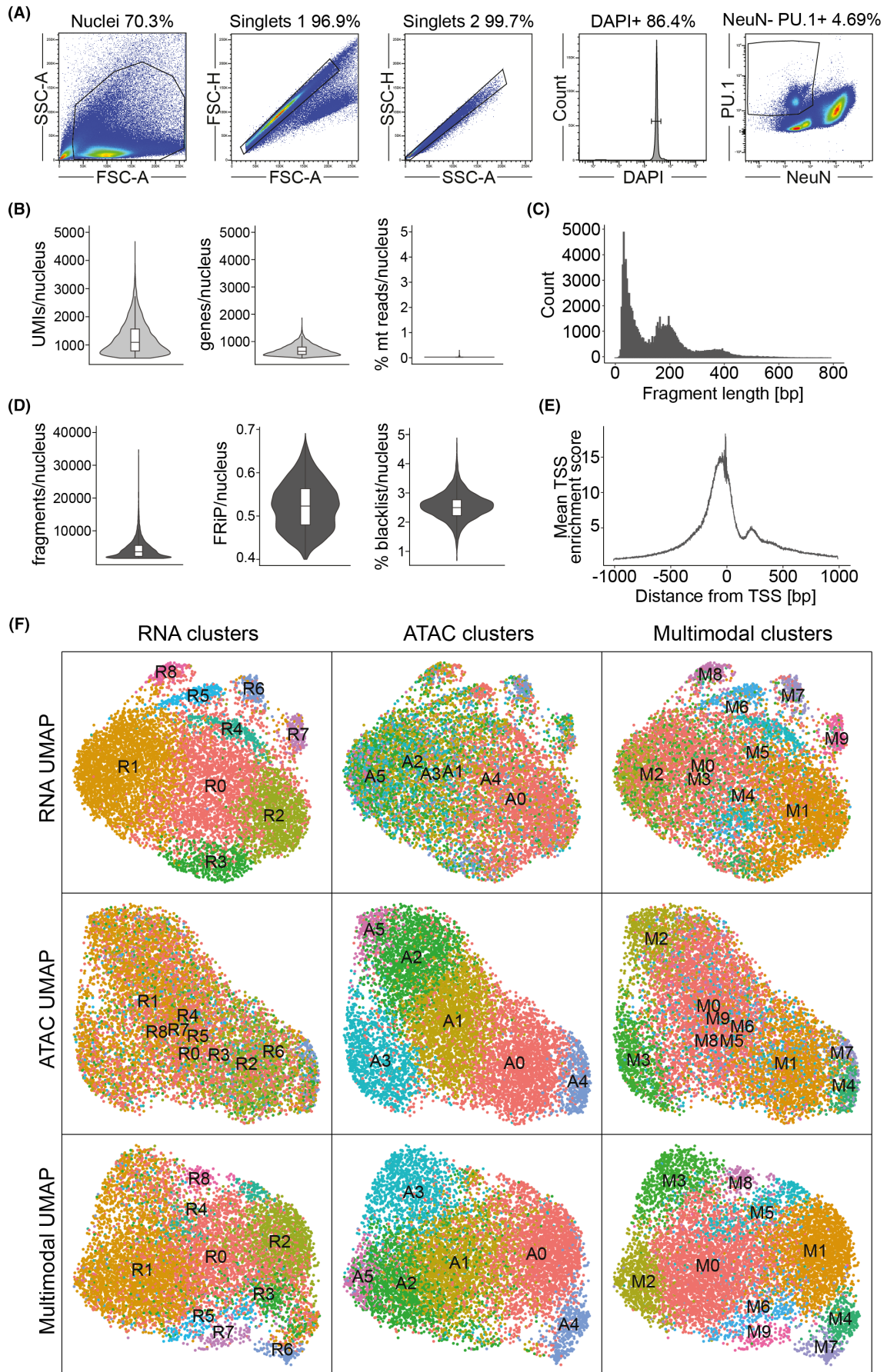
the multiome data set (Figure 2D). This initial observation indicates that joint analysis of gene expression and open chromatin may improve microglial subtype definition.

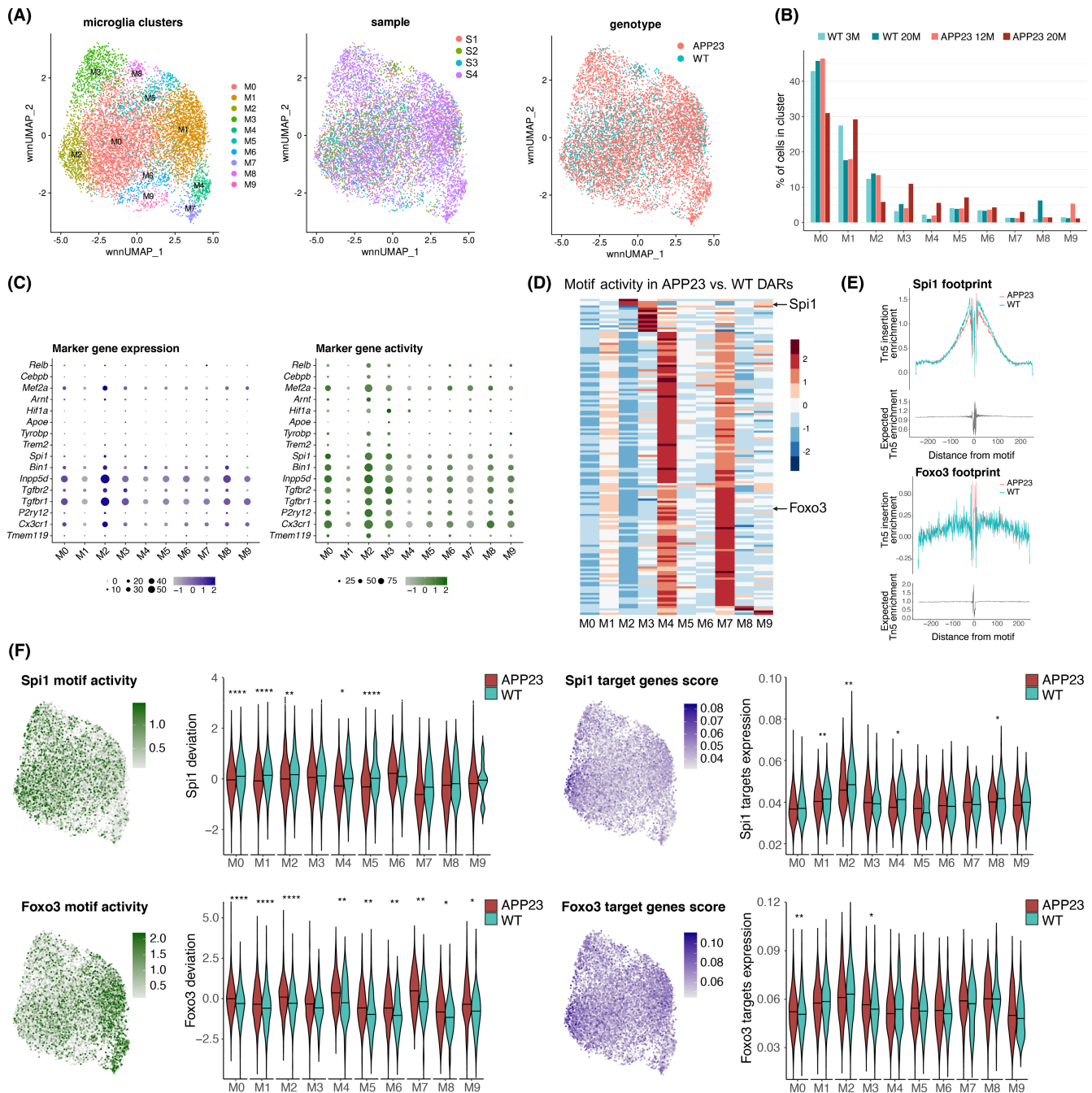
To study this further, we examined the effect of age and genotype on microglial subtype composition (Figures 3A,B), revealing age- and disease-associated shifts in microglia from 20-month-old wildtype and APP23 mice, respectively. In particular, we observed an increase in M8 microglia with age in wildtype animals while in APP23 animals, M3, M4 and M5 microglia increased proportionally; at the same time, microglia in the M0 and M2 clusters decreased in 20-month-old APP23 animals (Figure 3B). To gain a first impression of the possible advantages of performing multimodal analysis, we then examined expression of a selected set of marker genes for homeostatic and disease-associated microglia (DAM) and transcription factors highlighted in the studies reviewed above. We found that M0 and M2 clusters likely represent homeostatic microglia, showing high expression levels of *Cx3cr1*, *Tgfb1*, *Mef2a*, *Inpp5d*, *Bin1* and *Spi1*. Notably, gene activity levels (based on snATAC-seq peak signal throughout the gene body and promoter) better reflected other homeostatic gene activities, particularly those of *Tmem119* and *P2ry12*, which showed low transcript abundance. Similarly, although an increase in DAM markers such as *Trem2*, *Tyrobp*, *ApoE* and *Hif1a* and a concomitant decrease in the homeostatic markers *Tmem119*, *Cx3cr1*, and *P2ry12*, was evident at transcript level in M3 microglia, these changes were much more pronounced in the snATAC-seq data. While these results will need to be validated further, they indicate that inclusion of snATAC-seq profiling can compensate for transcripts with low abundance in the nucleus, which have been reported to contain several disease-associated microglial marker genes.<sup>121</sup>

Next, we asked whether our pilot multimodal data could corroborate some of the findings from the literature. We therefore examined gene expression and activity of *Cebpb*, which was recently reported to be increased in aged microglia.<sup>32</sup> While transcript levels of *Cebpb* were low in our data set, gene activity was detectable and highest in M8 microglia, which we found to be more abundant in microglia from aged, 20-month-old wild-type animals (Figure 3B), matching the previous report.<sup>32</sup> Similarly, *Bin1*, *Relb*, *Hif1a* as well as *Spi1* gene activities showed microglial subtype-specific modulation reflective of recent studies.<sup>20,33,50</sup> In particular, *Bin1* and *Spi1* were reduced and *Hif1a* gene activity was increased in the M3 (DAM) compared to the homeostatic M0/2 clusters, while *Relb* was increased in a distinct set of microglial subtypes (M2/6/9).

Our snATAC-seq data also allowed us to examine the effect of genotype, and therefore A $\beta$  pathology, on TF binding motif

**FIGURE 2** Multimodal analysis enhances microglial subtype classification. Microglial nuclei were isolated from frozen mouse brains and analyzed using an adapted SHARE-seq method.<sup>120</sup> (A) Flow cytometry strategy for purifying microglial nuclei (Pu.1+). (B) Number of transcripts, total genes, and mitochondrial genes per nucleus after exclusion of low-quality cells; (C) Fragment length; (D) Number of fragments, fraction of reads in peak (FRIP) score and % blacklist per nucleus; and (E) transcription start site (TSS) enrichment score for microglial nuclei in the analyzed data set. (F) Clustering analysis of 9327 microglial nuclear profiles based either on snRNA-seq or snATAC-seq data alone or on the integrated multimodal data set, with cluster overlay onto the other UMAP plots, demonstrates a significant impact on microglial subtype classification depending on the modality used.





**FIGURE 3** Multimodal analysis confirms age- and disease-associated microglial responses. (A) Microglial clustering analysis of SHARE-seq data (left panel, cp. Figure 2) color-coded by sample (demonstrating successful data integration of the four analyzed samples, S1-S4; middle panel) and by genotype (right panel). (B) Proportion of microglia across the different clusters in the four animals analyzed, indicating age- and A $\beta$  pathology-induced shifts in cell distribution. (C) snRNA-seq and snATAC-seq based analysis of gene expression (left panel) and gene activity (right panel), respectively, demonstrating higher sensitivity of snATAC-seq for certain marker genes, whose transcripts may have low cytoplasmic abundance. (D) Comparative analysis of TF motif representation in differentially accessible chromatin regions (DARs) from APP23 versus wildtype microglia, with (E) TF footprinting for Spi1 and Foxo3 and (F) their cluster-specific and genotype-dependent motif activity and target gene expression.

representation in differentially accessible chromatin regions. This analysis revealed several TF binding motifs involved in the microglial response to A $\beta$  pathology in APP23 animals. Interestingly, the largest numbers of modulated TF motifs were found in clusters M4 and M7; thus, these microglial subtypes may represent cells that are undergoing cell state transitions as they also did not show strong

gene expression/activity of homeostatic or DAM markers. However, this will require further analysis.

We selected two TFs overrepresented in differentially accessible regions (DARs) in microglia from APP versus wildtype animals, Spi1 and Foxo3, for further analysis as they have previously been implicated in microglial state transitions in response



to AD pathology.<sup>33,50,51</sup> Considering all open chromatin regions, we first used TF footprinting (i.e., shifts in Tn5 binding sites that result from TFs bound to open chromatin blocking Tn5 access and therefore transposition) to examine the overall level of TF activity in microglia from wildtype and APP animals. As expected, this revealed a clear Tn5 insertion enrichment for Spi1 and to a lesser extent Foxo3 (Figure 3E). Interestingly, in contrast to the increased activity of Spi1 in DARs, total Spi1 binding was reduced in microglia from APP23 animals. We therefore analyzed Spi1 motif enrichment in open chromatin regions and the expression levels of its predicted target genes (based on its motif in promoter regions) in a cluster and genotype-specific manner. Indeed, this confirmed that in microglia from APP23 animals, clusters M0, M1, M2, M4, and M5 showed overall reduced Spi1 motif accessibility, with Spi1 target gene expression also significantly decreased in clusters M1, M2, M4, and M8. However, the M3/DAM clusters showed similar levels of Spi1 motif activity and target gene expression in microglia from wildtype and APP23 mice. These data indicate that while most microglial subtypes show lower PU.1 signaling, PU.1 signaling is comparatively higher in the M3/DAM phenotype reflecting their response to A $\beta$  pathology, as previously reported.<sup>49–51</sup>

In contrast, in microglia from APP23 animals, Foxo3 motif enrichment was higher in all but the M3 cluster, indicating that it may be broadly activated in microglia responding to A $\beta$  pathology. Interestingly, Foxo3 target genes were only found to be increased in the M01 and M3 clusters, indicating that, as reported in human microglia,<sup>33</sup> Foxo3 may be required to trigger the transition to a proinflammatory activation state, which in mice may manifest as the DAM (M3) population. Again, this observation requires further validation with a higher number of biological replicates and across disease stages.

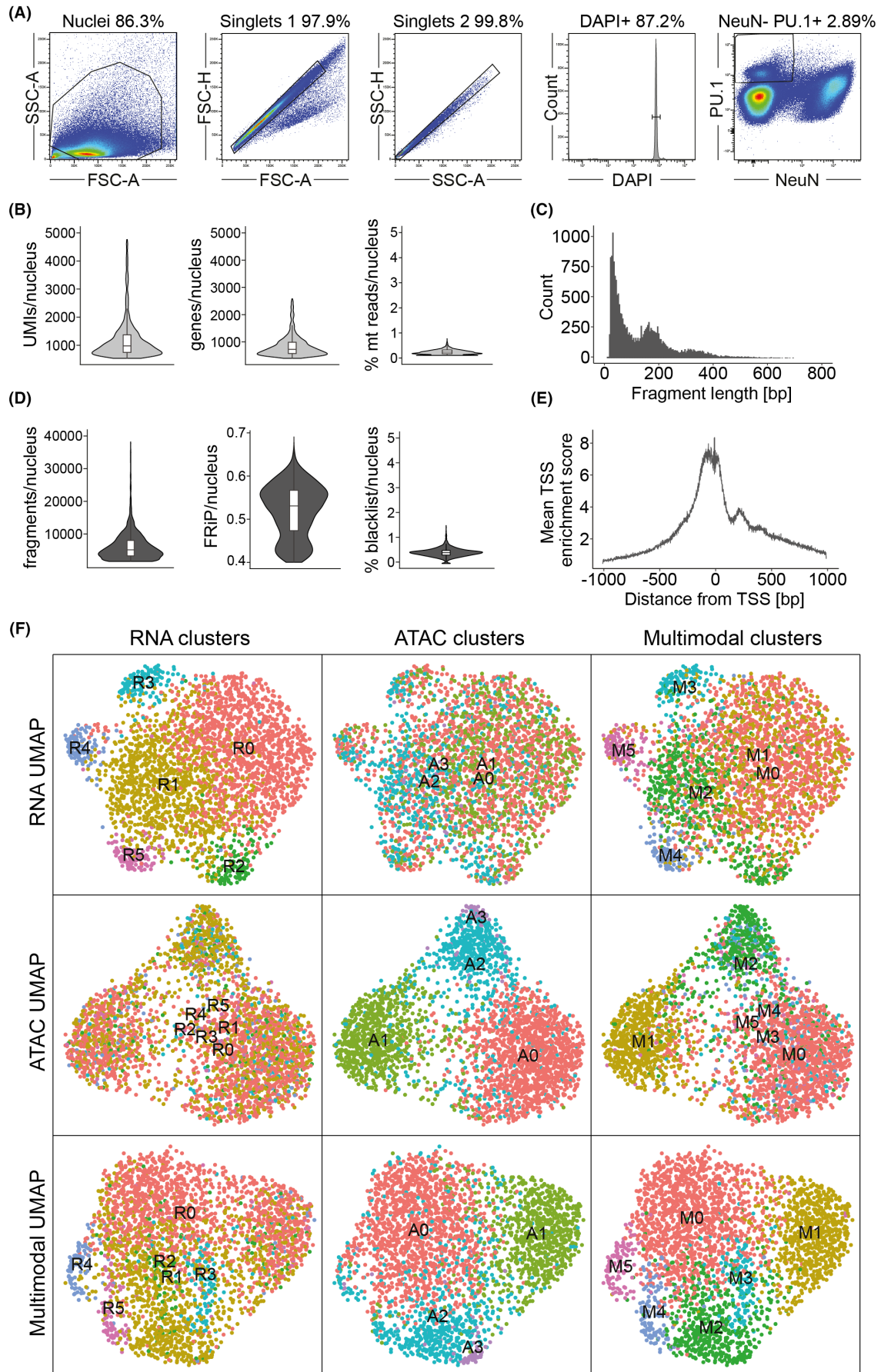
Finally, we also tested our adapted SHARE-seq protocol for analysis of human tissue using a frontal cortex sample from a nondemented, 87-year-old patient, who donated their brain ~8 years ago. Using the same approach as for fresh frozen mouse brains, we optimized conditions to enrich microglial nuclei and in a pilot experiment, retained 2971 microglial nuclei after quality control (Figure 4). Despite the extended storage period, data quality was comparable to mouse brains (Figures 4A–C), indicating suitability of this multimodal method for human microglia analysis. In line with our observations using mouse microglia, clustering based on snRNA-seq, snATAC-seq or multimodal data alone demonstrated that multimodal data analysis impacted microglial subtype assignment. In particular, snATAC-seq data only resolved 4 subtypes while snRNA-seq and multimodal clusters showed six subtypes each; however, cluster composition was noticeably different between transcriptomic and multimodal data (Figure 4D). Thus, future work will have to investigate how inclusion of epigenetic data (in particular, using multimodal analysis to allow for direct comparisons) may alter interpretation of previously transcriptionally-defined microglial subtypes in the healthy and diseased brain.

## 11 | DISCUSSION AND FUTURE PERSPECTIVES

Transcriptomic analyses are now being widely used to characterize macrophage and microglial phenotypes, with a plethora of studies describing novel subtypes of microglia under different pathophysiological contexts.<sup>122</sup> In contrast, only over the last few years have experimental studies started to include epigenetic analyses to understand the molecular responses and functions of tissue-resident macrophages, with a limited number of studies published on microglial cells so far. However, genome-wide epigenetic profiling of microglia has already led to several important insights, including epigenetic control of brain region-specific microglial functions,<sup>59</sup> mapping of disease-associated variants to microglial enhancers,<sup>15,51</sup> and revealing microglial innate immune memory function.<sup>18,87</sup> Recent technological and methodological developments will enable epigenetic profiling at high throughput in the coming years, and we expect that these approaches will contribute significantly to our understanding of microglial activation states and function.

For instance, while the epigenetic underpinnings of specific microglial functions in the cerebellum have been shown in mice,<sup>59</sup> similar mechanisms remain to be investigated for other brain regions and for different brain compartments (e.g., white and gray matter and the brain's vasculature) both in model systems and in the human brain. Moreover, as outlined and demonstrated above, it is conceivable that joint epigenetic and transcriptomic profiling may lead to a reclassification of microglial subtypes as identified by single-cell sequencing analyses, given that even in our small pilot dataset the inclusion of epigenetic profiles had a noticeable effect on microglial subtype assignment. This may be because epigenetic changes do not only reflect the acute macrophage response but often foreshadow transcriptomic changes, in particular, enhancer activation often precedes gene expression.<sup>45,123</sup> Therefore, changes in the epigenetic profile of microglia may provide biologically important indicators of their transition into other activation states, which may be resolved, for example, with recently developed software tools that utilize both epigenomic and transcriptomic data to predict cellular differentiation/activation trajectories.<sup>124</sup> Analysis of such transitional states will not only provide us with a better understanding of the molecular mechanisms that drive microglia responses but may also reveal novel molecular targets to promote or prevent the induction of specific microglial phenotypes, with important implications for the therapy of neurological diseases.

Similarly, epigenetic profiling of microglia may delineate how multiple insults affect the brain's immune responses, with particular importance for human brain health, as humans are exposed to a variety of immunological insults in their lifespan. While it has been reported that mouse and human macrophages as well as mouse microglia can integrate multiple insults at the epigenetic level, thereby modifying their future responses,<sup>18,66,80,87</sup> if and how this happens in the human brain remains unresolved. Moreover, while





**FIGURE 4** Multimodal analysis of human microglia. Microglial nuclei were isolated from frozen human brain tissue from an 87-year-old donor, ~8 years after brain donation. The protocol for microglial nuclei isolation was equivalent to mouse tissue processing and nuclei were again analyzed using our adapted protocol for the SHARE-seq method.<sup>120</sup> (A) Flow cytometry strategy for purifying microglial nuclei (Pu.1+). (B) Number of transcripts, total genes, and mitochondrial genes per nucleus after exclusion of low-quality cells; (C) Fragment length; (D) Number of fragments, fraction of reads in peak (FRiP) score and % blacklist per nucleus; and (E) transcription start site (TSS) enrichment score for microglial nuclei in the analyzed data set. (F) Clustering analysis of 2971 microglial nuclear profiles based either on snRNA-seq or snATAC-seq data alone or on the integrated multimodal data set, with cluster overlay onto the other UMAP plots, demonstrating a significant impact on microglial subtype classification depending on the modality used.

this review is focused on microglia, it will be equally important to study epigenetic regulation in other brain cell types. Thus, we expect that inclusion of epigenetic analyses will provide exciting insights into the complexity of brain development, health, and disease beyond what current omics analyses have been able to reveal.

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### CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

### DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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