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Review: Microglia in motor neuron disease

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B. A. Ashford, D. Boche, J. Cooper-Knock, P. R. Heath, J. E. Simpson and J. R. Highley (2020) *Neuropathology and Applied Neurobiology* **Review: Microglia in motor neuron disease**

Motor Neuron Disease (MND) is a fatal neurodegenerative condition, which is characterized by the selective loss of the upper and lower motor neurons. At the sites of motor neuron injury, accumulation of activated microglia, the primary immune cells of the central nervous system, is commonly observed in both human *post mortem* studies and animal models of MND. Microglial activation has been found to correlate with many clinical features and importantly, the speed of disease progression in humans. Both anti-inflammatory and pro-inflammatory microglial responses have been shown to influence disease progression in humans and models of MND. As such, microglia could both contribute to and protect against inflammatory mechanisms of pathogenesis in MND. While murine models have characterized the microglial response to MND, these studies have painted a complex and often contradictory picture, indicating a need for further characterization in humans. This review examines the potential role microglia play in MND in human and animal studies. Both the pro-inflammatory and anti-inflammatory responses will be addressed, throughout the course of disease, followed by the potential of microglia as a target in the development of disease-modifying treatments for MND.

Keywords: humans, inflammation, microglia, motor neuron disease

Motor neuron disease

Motor Neuron Disease (MND) is a fatal neurodegenerative condition characterized by the progressive degeneration of both upper motor neurons in the motor cortex, and the lower motor neurons in the brain stem and spinal cord [1]. MND has a number of subtypes, of which Amyotrophic Lateral Sclerosis (ALS) is the most common and affects around two in every 100 000 people [2]. Throughout much of the literature, ALS and MND are used synonymously; here MND will be used to refer to all subclasses of the disease.

Typically, MND presents with progressive muscle weakness in the extremities, muscular atrophy, fasciculations, cramping, spasticity and fatigue [1]. Diagnosis of MND normally occurs around 55–60 years of age. While survival time varies from just a few months to decades [3], 80% of patients survive only two to five years after diagnosis, with death usually resulting from paralysis and respiratory failure [1]. Currently there is no cure, and the only US Food and Drink Administration-approved treatments, Riluzole and Edaravone, have modest effects, extending survival by mere months [1].

MND is pathogenically complicated, and the cause of MND in most cases is unknown: 90–95% of cases are sporadic (sMND), while the remaining 5–10% are familial (fMND), generally with autosomal dominant inheritance [4]. More than forty pathogenic mutations have been associated with MND, including mutations in *SOD1* (*Superoxide dismutase*) [5], *TARDBP* which encodes Transactive Response DNA-binding protein 43 kD (TDP-43) [6], *FUS* (*RNA Binding Protein Fused in*

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Sarcoma/Translocated in Sarcoma) [7,8], and the most common, *C9orf72* (*chromosome 9 open reading frame 72*) [9]. Interestingly, a number of MND-associated genes have a direct role in immunity, such as *TBK1* [10], *OPTN* [11], *VCP* [12] or pathways that are important for immune function, such as the autophagy and lysosomal pathways (e.g. *SQSTM1* [13], *UBQLN2* [14]).

Neuropathology of MND

There are many pathological changes associated with MND including depletion of giant pyramidal (Betz) cells and atrophy of the precentral gyrus, thinning of the spinal ventral roots, sclerosis and pallor in the corticospinal tracts and loss of lower motor neurons [15].

The hallmark microscopic pathology of MND is the presence of protein aggregates in the remaining motor neurons. In the majority of cases, these aggregates are composed of ubiquitylated TDP-43 [6]. However, aggregates may also include or be composed of other proteins including FUS and SOD1 [16]. TDP-43-positive inclusions are primarily seen in the motor structures of the spinal cord, corticospinal tract and the motor cortex [17]. These inclusions are also observed to a lesser and more variable extent in extramotor regions including the anterior frontal and temporal cortices [18]. MND is considered to be on a disease spectrum with Frontotemporal Dementia which is characterized by changes in personality and behaviour, loss of language skills and cognitive decline [19]. Along this continuum cases show pathology purely in motor regions, ranging to FTD cases with purely nonmotor region pathology [18]. This spectrum is reflected clinically with some MND patients displaying purely motor symptoms, some patients having both motor and cognitive or behavioural decline and some FTD patients without motor symptoms.

Glial involvement in MND

In models of MND, motor neurons show dysfunction in many cellular processes including axonal transport and microtubule function [20,21], RNA processing [22], endoplasmic reticular stress response [23] and endosomal trafficking [24]. However, MND is not a purely neuron-autonomous disease; glial cells are highly involved and also display inclusion pathology both in human MND and in animal models [25,26]. Microgliosis and astrogliosis, the emergence of activated microglia and astrocytes respectively, can be seen in human *post mortem* tissue and animal models [27,28]. Further, in a chimeric mutant $SOD1^{G93A}$ mouse model, mutant SOD1 (mSOD1) expressing motor neurons showed improved survival when surrounded by wild-type astrocytes and microglia, and in contrast, wild-type motor neurons showed MND pathology when surrounded by mSOD1 glia [29], indicating that the glial environment influences neuronal pathology in MND. While infiltrating immune cells, such as mast cells, T cells, dendritic cells and macrophages, are also a feature of MND [27,30,31], the remainder of this review will focus on the complex role microglia play in the progression of MND.

Microglia – the primary immune cells of the central nervous system

Microglia are the resident immune cells of monocytic lineage and are the primary form of immune defence within the central nervous system (CNS). These highly active cells have several functions in development, homeostasis and innate immunity [32].

Under homeostatic conditions, microglia have a ramified morphology with small somata and short, fine processes that constantly extend, retract and reform in order to survey the local microenvironment [33]. Under pathological conditions, microglia have functions in pathogen destruction, the resolution of inflammation and the promotion of healing and cell growth. Microglia react to signals from potential pathogens (Pathogen-Associated Molecular Patterns; PAMPs), and signals expressed by damaged endogenous cells (Damage-Associated Molecular Patterns; DAMPS), via the activation of pattern recognition receptors [34]. In response, microglial cytoplasmic processes swell and shorten, becoming less ramified, and at the extreme end of the spectrum, the cell can adopt a large, 'amoeboid' morphology (Figure 1). This activation is associated with alterations in the molecules that they express. Once the activating stimulus has been resolved, microglia may revert to their homeostatic ramified state.

However, with ageing and chronic activation microglia have been observed to adopt another phenotype, often referred to as the dystrophic phenotype [35,36] .

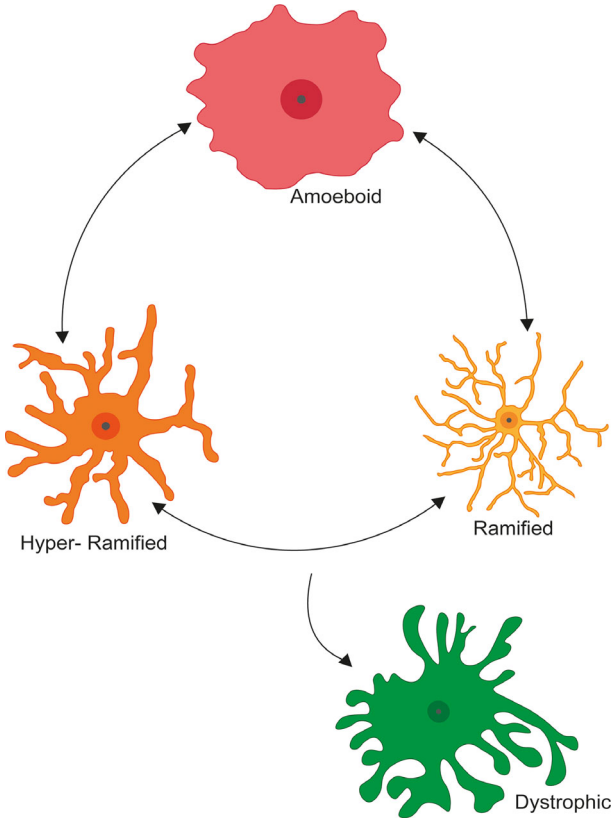

Figure 1. Microglial morphology. In their resting state microglia have a ramified morphology. Once activated, their cytoplasmic processes swell such that the cell ultimately adopts a large amoeboid morphology. Upon resolution of the activating stimulus, microglia revert to their ramified state. With aging microglia can adopt a dystrophic phenotype characterised by loss and beading of the processes and fragmentation of the cytoplasm.

These cells show reduced surveillance and dendritic branching [37], a redistribution of Iba1- particularly in the processes [38] and reduced motility and migration [39]. Furthermore, these cells show changes in gene expression including expression of pro-inflammatory cytokines in homeostatic conditions [40] and more sustained responses to damage [39], as well as storage of iron as ferrate [41]. As this phenotype is most commonly observed in the ageing brain, this dysfunctional phenotype may represent microglial senescence.

Microglia present a multifaceted inflammatory response. This includes the release of pro-inflammatory cytokines and reactive oxygen and nitrogen species to aid the destruction of pathogens and damaged cells. Chemokines may be released to further activate and recruit other glial and immune cells to the site of injury [32]. Microglia also have functions relating to inflammation mediation and resolution, debris clearance, tissue healing and growth. This is achieved firstly, via the release of anti-inflammatory cytokines and trophic factors, which promote healing, repair and tissue remodelling. Secondly, they may increase the number of surface scavenger receptors to promote pathogen and debris phagocytosis [42].

Historically, activated microglia have been classified as pro-inflammatory (M1) or anti-inflammatory (M2). However, this binary system has received criticism. It has been suggested that activated microglia may fall on a spectrum, with some cells being purely M1 through to some cells which show a purely M2 phenotype [43,44]. However, transcriptomic profiling of *ex vivo and in vivo* microglia isolated from mouse models of various neurodegenerative conditions, failed to find evidence of an M1/M2 spectrum, with many microglia exhibiting disease-specific profiles [45,46]. Furthermore, single-cell-based analyses of microglia from both mouse and human cortex have revealed several transcriptionally distinct phenotypes which differ from the M1/M2 phenotypes [47,48]. This said, a lot of the literature still follows this type of characterization and uses classic inflammatory markers to determine microglial function. As such, in this review, we will focus on microglial functions and will use the terms pro-inflammatory and anti-inflammatory to broadly describe microglial activation. However, these terms are used with caution and the reader is respectfully advised to bear in mind that this is done as a 'shorthand' to ease discussion. We do not wish to imply that we endorse a simple binary classification.

A brief summary of some of the main molecules associated with microglia is given in Table 1. Microglial phenotype is affected by a number of complex factors including location within the CNS [49,50], microglial age [51], activating stimuli and disease [47], as well as well as differences between human and murine microglia [51]. Furthermore, microglia may express molecules with pro-inflammatory and anti-inflammatory functions simultaneously. Therefore, it should be noted that Table 1 is not a comprehensive list and is focused more on findings related to neurodegenerative disease, particularly MND.

Microglial activation is effective in the short-term removal of damaged cells and pathogens and is necessary for the protection of the CNS. However, where

Table 1. Summarization of microglial phenotypes, their activating stimuli and typical markers for their identification

Associated functions	*Homeostatic state and pathogen detection*	*Pathogen destruction- associated with inflammation*	*Inflammation suppression and phagocytosis*	*Tissue remodelling and extracellular matrix deposition*
Upregulated cytokines		TNF-α, IL-1β, IL-1α, IL-6, IL-12a, IL-12b, IL-15, IL-18, **IL-23**, IL-33, IFN-γ	**IL-4,** IL-10, **IL-13 ARG1*, TGF-β**, **FIZZ1***	IL-10
Chemokines		CCL1, CCL2 (MCP-1), CCL3, **CCL4,** CCL5, **CCL8**, CCL12 (MCP-5), **CXCL2**, **CXCL4,** CXCL9, CXCL10, CXCL13	CXCL16, **CCR2 (CD192),**	CXCL13
Enzymes	HEXB+,	NOX2 (CYBB), NOS2, iNOS, CASP1 (ICE), **COX2**		MMP-12, **ARG1*?, YM1*?**
Receptors and Transmembrane Proteins	TGFBR1+, P2RY12+, P2RY13+, FCRLS*, TMEM119+, GPR34+, SIGLEC-H+, CD11b (ITGAM), Iba1(AIF1*), CXC3R1, CSF1R	MHC II receptors including HLA-DR, IFNar1, IFNar2, CD45, CD119 (IFNgr1), CD14, TLR-2, **CCR8**,	CD163, CD204, IFNAR1, IFNAR2, IL-10RA, CD68, IFITM3, CD16 (FcγRIII), CD32 (FcγRII), **CD33**, CD64, TREM2*?	MHCII, **CCR8**
Growth factors			IGF1	BDNF
Transcription and signalling factors	SALL1+, PU.1,	IRF1, **STAT3**		
Other	OLFML3+, GLUT5, ROS	S100A8, S100A9, IRAK1	OPTN, DAP12 (TYROBP), GRN,	
References	[47,142–145]	[32,47,145–151]	[47,145–147,151]	[32,47,146,151–153]

Key: mouse specific*; Human expression uncertain*?; **Result from In-vitro study;** Microglial Marker/Signature Gene+

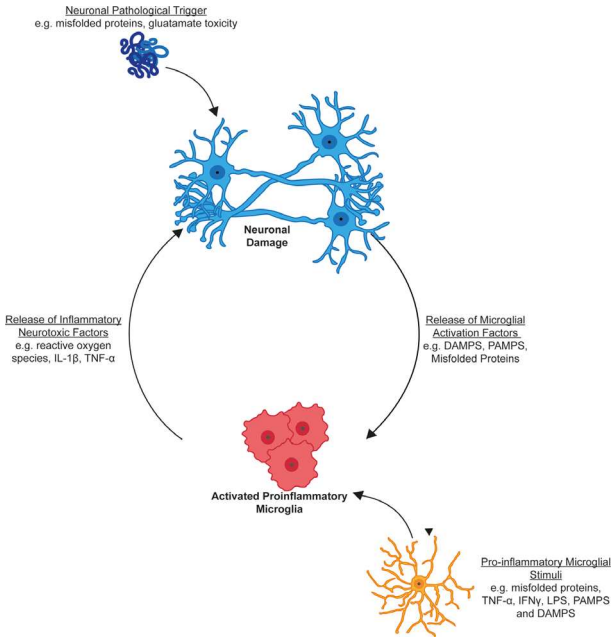

Figure 2. The cycle of microglial/ neuronal neurotoxicity. The toxicity cycle can be started in two ways. First, an external stimulus directly activates microglia, which produce cytotoxic factors that cause neuronal damage. Secondly, neuronal damage can be initiated by another pathological trigger, which results in the release of DAMPs, which in turn activates microglia, resulting in a toxic environment for other neurons (reactive microgliosis). This results in a self-perpetuating cycle of microglial over-activation and neuronal damage.

the initial trigger is not resolved, a destructive cycle of microglial activation and neuron death is initiated (Figure 2). Briefly, this cycle can be triggered by PAMPs causing microglial cytotoxicity leading to neuronal death. Alternatively, if neuron death is triggered by processes such as protein misfolding and aggregation, or excitotoxicity, DAMP release can cause cytotoxic microglial activation with increased inflammation and neuronal death [52]. This process is seen as a driver of progression in neurodegeneration [52,53].

Microglial activation in human MND

Human studies have demonstrated the importance of microglia in MND. *Post mortem* spinal cord from MND patients has been found to contain raised levels of Monocyte Chemoattractant Protein 1 (MCP-1; more commonly known as C-C Motif Chemokine Ligand 2 or CCL2), a key recruiter of microglia [31]. At sites of neuronal loss, immunohistochemistry for key markers of microglia/macrophages, Cluster of Differentiation (CD) 68 and Ionized calcium binding adaptor molecule 1(Iba1), reveals microglia that have transitioned from their ramified morphology to the 'activated' morphology described above [31,54,55].

A population of microglia, which are immunopositive for the haemopoetic transmembrane phosphoglycoprotein, CD34, have been observed in the ventral horn of sMND spinal cord [56]. In control cases, immunoreactivity for CD34 is restricted to the blood vessels and capillaries. However, in MND cases, CD34+ cells were found interspersed throughout the ventral horn forming clusters around motor neurons. These cells showed a rounded morphology, with a small subset staining positively for the microglial/macrophage marker Iba1. These cells were also Ki67+, indicating that these cells may represent an alternative, proliferative microglial phenotype.

The presence of activated microglia is associated with a number of key pathological and clinical features of MND. Increased microglial activation has been observed in the key sites of neuronal degeneration, as indicated by increased expression of CD68 and Iba1 in *post mortem* motor cortex and spinal cord, compared to control [54]. This has also been observed to a lesser extent in nonmotor regions including the amygdala, hippocampus and the middle frontal and superior temporal gyri [54]. Furthermore, the presence of $Iba1^{+}$ microglia correlated with the extent of neuronal loss in the motor cortex, spinal cord and middle frontal gyrus, while increased microglial activation in the middle frontal cortex and superior temporal gyrus was associated with impaired executive function [54].

Additionally, increased microglial activation, as illustrated by CD68 and Iba1 immunostaining, in the corticospinal tract has been significantly associated with more rapid disease progression [55]. Familial cases associated with *C9orf72* mutations tend to show more extensive microglial pathology within the corticospinal tract [55]. Reactive microgliosis has also been described in MND cases associated with other disease-causing mutations such as *TBK1* [57], *SOD1* [58], *FUS* [59], and *ANG* [60]. Furthermore, higher scores on a clinical upper motor neuron assessment scale, based on the Ashworth Spasticity Scale, are associated with

increased microglial pathology in the upper corticospinal tract. [55]

While such autopsy studies represent end-stage disease, these findings are supported by *in vivo* Positron Emission Tomography imaging, where neuroinflammation in MND was observed using the 18-kDA translocator protein (TSPO) PK11195 tracer ligand [61]. The binding of this ligand is highly associated with activated microglia but has also been observed in other cell types including astrocytes, and is associated with neuroinflammation. In comparison with neurologically healthy individuals, MND patients showed increased signal in both motor regions and extramotor regions. A further PET study showed neuroinflammation in MND to be associated with cortical thinning and a worse disease phenotype as indexed by the ALS Functional Rating Scale [62]. Caution is required in interpretation of these results due to the lack of specificity of the marker.

Studies of a microglial regulatory protein, Triggering Receptor Expressed on Myeloid cells 2 (TREM2) have also implicated microglia in MND. TREM2 is a membrane-bound protein, which is expressed by multiple myeloid cell populations including microglia, and promotes myeloid activation and phagocytosis, and downregulates the transcription of pro-inflammatory cytokines [63]. Soluble TREM2, a cleaved form of the protein, was found to be upregulated in human MND patient spinal cord and associated with improved patient survival [64,65]. Furthermore, soluble TREM2 mRNA has been found to be highly expressed in cerebrospinal fluid (CSF) in the early stages of MND and diminished in later stage disease [65]. In the late stages of disease, *TREM2* expression correlated with survival time in MND patients, perhaps reflecting a continued neuroprotective response [65]. A rare *TREM2* missense variant (p.R47H) that dampens microglial neurotoxicity has been identified as risk factor for neurodegenerative conditions including Alzheimer's disease (AD) [66] and potentially MND, although results have varied [64,67].

A recent study by Zhou *et al.* [68] has shown that *TREM2* has a key role in regulating disease-associated microglial gene expression in both human AD and in mouse models of AD using single nuclear RNA sequencing, supporting the role of *TREM2* in microglial gene expression regulation. However, microglial gene expression differed greatly in humans compared to mouse models of AD, indicating a species-specific role for *TREM2* in microglial regulation. Furthermore, while TREM2 is highly associated with microglial activation and regulation, the expression of TREM2 by human microglia has recently been questioned; TREM2 immunohistochemistry on human cortical tissue showed that TREM2 was overwhelmingly expressed by infiltrating monocytes, while little expression was observed in native microglia [69]. Such species-specificity stresses the need for human as well as animal research.

Cerebrospinal fluid from MND patients has shown high levels of inflammatory cytokines including TNF-α, IL-1β and IFN-γ [70–72]. Furthermore, microarray transcriptomic analysis of human spinal cord grey matter has shown an increase in inflammatory signalling in MND, characterized by various interleukins and cathepsins [73].

In summary, human pathology, imaging and genetic studies have supported a role for microglia in MND. The small amount of published research that attempts to determine specifically which microglial functions are relevant in human MND favours an inflammatory process.

Microglial function in animal models of MND

Attempts to determine the molecular behaviours of microglia in MND with greater specificity have predominantly utilized transgenic animal models. The first made, and most widely utilized and characterized MND model was generated in mice using the familial G93A mutation in the *SOD1* gene [74]. Further mSOD1 models have been developed using other patient variants [75–77]. The mSOD1 models rely on high transgene copy numbers, which produces a widespread overexpression of mutant protein, resulting in a reliable but severe disease phenotype [78]. While each variant differs in terms of the age of disease onset, rate of disease progression and symptom presentation, generally the mSOD1 models show extensive MN loss, aggregates of misfolded protein, progressive paralysis and shortened life span [79].

Following this, models have been created which contain transgenic mutations in *TARDBP*, *FUS*, *C9orf72*, *PFN1* and *UBQLN2* (summarized by Lutz [79]), as well as in other species including rats [80]

and zebrafish [81–83]. The MND models replicate many of the key features of disease and have provided a mechanism to study the progression of MND pathology. However, these models often show more aggressive disease progression compared to the human disease, and as it is the case with all disease models, no one model is able to replicate all disease features [78].

Microglia in early phase disease in animal models.

Research to ascertain whether microglial changes are involved in the initial stages of pathology in murine models of MND have reported conflicting findings. mSOD1^{G93A} mice show evidence of peripheral nerve and motor neuron dysfunction early, before the appearance of CD68^{+} macrophages and microglia in the muscle and spinal cord respectively [84]. In contrast, decreased levels of Iba1^{+} microglia in mSOD1^{G93A} mice spinal cord, prior to symptom onset have also been reported [85]. Furthermore, no change in microglial number was observed in mSOD1^{G93A} rat spinal cord isolated at the equivalent presymptomatic stage [28]. However, a significant increase in the number of microglia was observed immediately postsymptom onset [28]. These mixed findings make it difficult to determine whether microglia are actively involved in the initial motor neuron pathology, or whether neuroinflammation is secondary to motor neuron pathology. Given the microglial functions associated with CD68 (phagocytosis) and Iba1 (motility), these data could suggest that microglia may be recruited to the sites of neuronal pathology before the appearance of symptoms, whereas the CD68+ microglia may reflect microglia responding to the damage already present. Additional phenotypic investigation of microglia in MND is required to characterize their role in humans. In the later disease stages, animal models have tended to focus on the trophic and anti-inflammatory, versus neurotoxic or pro-inflammatory effects of microglia. These roles will now be considered in turn.

Anti-inflammatory and trophic microglia in animal models of MND

In the early stages of most diseases, microglia are activated by signals from damaged or stressed neurons, and potential pathogens [86]. In MND, these signals are likely to include various chemokines and DAMPS released by motor neurons, and the uptake of misfolded protein aggregates by microglia [87,88]. Transcriptomic analysis of microglia isolated from 11-week-old mSOD1^{G93A} mice has shown high levels of *Ym1*, an anti-inflammatory macrophage marker, as well as Brain-derived Neurotrophic Factor (BDNF), a neurotrophin which promotes neuronal survival [44]. Interestingly, these microglia were also found to express increased mRNA transcripts for CD163, a haptoglobin-haemoglobin scavenger receptor predominantly expressed by perivascular macrophages and by microglia, following breakdown of the blood-brain barrier [89], indicating this process could occur early in disease. Coculture of these microglia with wild-type motor neurons promotes neuronal survival, but only in the presence of astrocytes, potentially indicating a neuroprotective microglial response and a possible interdependence with astrocytes – however further evidence is needed to validate the microglial/astrocytic interaction.

A further study of microglia from presymptomatic mSOD1^{G93A} mice has demonstrated a 16-fold increase in IL-10 production, a cytokine responsible for inflammatory cytokine downregulation [90]. Conversely, targeting IL-10 overexpression in the spine resulted in delayed symptom onset and extended survival, while blocking IL-10 receptors using the mIL-10R1 antibody results in rapid precipitation of symptoms. Increased IL-10 expression is associated with reduced TLR-2 activity, which promotes microglial inflammatory cytokine production [91].

Together, these results indicate that microglia, in the presymptomatic stages of MND may counteract or prevent the inflammatory response associated with later disease stages potentially delaying symptom onset.

Pro-inflammatory microglia in animal models of MND

Transcriptional analysis of 11-week-old mSOD1^{G93A} mouse spinal cord homogenates has demonstrated a significant upregulation of inflammatory genes including cathepsin D and TNF-α [92]. This was thought to represent an inflammatory response in the presymptomatic stage. However, symptom onset has been observed at as early as 8.5 weeks of age in this model [85], implying that the inflammatory microglial phenotype observed actually occurs in the early,

postsymptom-onset stage of disease. Furthermore, these results contradict other findings of increased anti-inflammatory cytokine expression at the same age in the same mSOD1^{G93A} model [44].

The latter study went on to examine late-stage mSOD1^{G93A} mice and found a shift towards diminished anti-inflammatory markers and high levels of nitric oxide, a toxic immune mediator. Coculture of these late-stage microglia with wild-type motor neurons induced neuronal death [44], likely by increasing motor neuron sensitivity to glutamate, potentially resulting in excitotoxicity [93], a known feature of motor neuron pathobiology in MND. These microglia also appear to inhibit the protective uptake of glutamate by astrocytes in culture [93].

Microglia expressing mSOD1 appear to be more neurotoxic than wild-type microglia. Upon stimulation with the neurotoxicity-inducing PAMP, Lipopolysaccharide (LPS), microglia overexpressing mSOD1^{G93A} released more NO and superoxidase, than both microglia overexpressing wild-type *SOD1*, and wild-type microglia [94,95]. These mSOD1 microglia induced more neuronal death in coculture with motor neurons than their wild-type counterparts.

Interestingly, Weydt and colleagues [96] performed a similar experiment, but compared primary microglia from 3-day-old mice and 60-day-old mice (early symptomatic). Upon stimulation with LPS, only the adult mSOD1^{G93A} microglia showed a significant difference from the wild-type control, with increased *TNF-α* production and decreased *IL-6* expression. This suggests that adult and not perinatal mSOD1-expressing microglia are more neurotoxic than wild-type microglia.

This heightened response by aged mSOD1 microglia may reflect microglial priming, where microglia display an exaggerated response to stimuli after prior activation, such as exposure to mSOD1 [97]. Microglial priming is a feature of ageing and other neurodegenerative conditions such as Alzheimer's disease [98]: these microglia are less responsive to anti-inflammatory agents, promoting chronic inflammation [98].

Furthermore, transplantation of wild-type microglia into mSOD1^{G93A} mice, via bone marrow transplant, results in reduced MN loss and extended survival [95], indicating that in mSOD1 mice, treatment with wild-type microglia is sufficient to benefit motor neurons and consequentially mouse survival.

Further evidence of microglial toxicity in MND murine models comes from experiments with *CX3CR1* gene manipulations. *CX3CR1* encodes the receptor for the chemokine fractalkine. Together, this ligand and receptor mediate neuron-microglial interactions and protect neurons by preventing pro-inflammatory microglial activation [99]. The V249I variant of *CX3CR1* is associated with faster progression and shorter survival in human sMND [100]. Transgenic mSOD1^{G93A} bred with *CX3CR1*$^{-/-}$ mice produced a worsened MND phenotype with enhanced inflammatory microglial activation and decreased neuronal density compared to *mSOD1*/*CX3CR1*$^{+/-}$, mSOD1 mice and wild-type mice [101].

Interaction of microglia and MND-associated proteins

A number of the proteins associated with misfolding in MND appear to influence microglia. Phagocytosis of extracellular mSOD1 has been shown to cause pro-inflammatory activation of microglia [102]. On incubation with mSOD1 aggregates, primary mouse microglia adopted the amoeboid morphology. Real-time quantitative PCR (RT-qPCR) revealed increased expression of the inflammatory factors *TNF-α*, *IL-1β*, *NADPH oxidase 2 (NOX2)* and *superoxidase*, and a concomitant decreased expression of the repair factor *IGF-1*. When cocultured with primary mSOD1^{G93A} mouse motor neurons, these primed microglia proved to be neurotoxic, compared to un-primed mSOD1^{G93A} microglia. However, incubation of mSOD1 aggregates with motor neurons alone had no significant effect on motor neuron survival, indicating that extracellular mSOD1 is not directly toxic to motor neurons alone, and activation of microglia to a pro-inflammatory phenotype is required for neurodegeneration. Motor neuron death could be attenuated, by using antibodies to block TLR-2 and 4, and CD14, indicating a potential TLR-CD14-mediated pathway by which mSOD1 is able to induce the neurotoxic microglial phenotype.

Similarly, mutant TDP-43 (*mTDP-43*) has been shown to induce an exaggerated pro-inflammatory microglial phenotype characterized by *TNF-α*, *IL-1β* and *NOX2* in primary mouse microglia, compared with wild-type treated with TDP-43 or nontreatment [103]. Furthermore, the expression of mTDP-43 by microglia produces a more pro-inflammatory response to LPS stimulation compared to wild-type microglia *in vitro*

[104]. mTDP-43 appears to activate microglia through the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway [103]. The NF-κB pathway is a master regulator of inflammation, and is highly upregulated in MND patient spinal cord, and mSOD1 mice [104,105]. When activated, the NF-κB complex relocates to the nucleus, inducing further pro-inflammatory cytokine and chemokine expression [106]. The NF-κB pathway can be induced directly via pathologically truncated wild-type TDP-43 and even more potently by mTDP-43 in primary mouse microglia [103]. This results in the production of pro-inflammatory cytokines [103,105]. Additionally, NF-κB-activated mSOD1 microglia have been shown to attack healthy synapses *in vitro*, causing axonal damage, a feature not observed in wild-type microglia [105].

Thus, the accumulation of pathological, misfolded SOD1 and TDP-43 may contribute to a switch in microglial phenotype from an initially anti-inflammatory response to a pro-inflammatory phenotype.

Interestingly, recent research has indicated that dysfunctional microglia may contribute to the pathological translocation of TDP-43 from the nucleus to the cytoplasm. Using a transgenic zebrafish model, which coexpresses green fluorescent protein-tagged TDP-43 and microglia which express mCherry, *Svahn et al.* [107] visualized the nucleocytoplasmic transmission of TDP-43 and microglial dynamics *in vivo*. After initiating neuronal death by ultraviolet light, microglia rapidly approached and phagocytosed damaged neuronal somata as well as TDP-43 granules in the nucleus. Microglia were then depleted using a morpholino targeted at the macrophage transcription factor, *PU.1*. Ultraviolet light treatment in the absence of functional microglia resulted in the translocation of TDP-43 from the nucleus to the cytoplasm, a key feature of MND pathobiology. This quite artificial model of neuronal death indicates that dysfunctional microglial phagocytosis may contribute to the typical pathological translocation of TDP-43.

Finally, as noted above, hexanucleotide expansions in *C9orf72*, the most common genetic cause of MND, are associated with reduced expression of the C9orf72 protein. RNA sequencing and mass spectrometry have led to the suggestion that microglia have the highest C9orf72 levels of any cell type in mice [108,109]. In contrast, another study suggested that there are lower C9orf72 levels in microglia than neurons. This study used a mouse strain which carried a *LacZ* reporter in the *C9orf72* orthologue, which was detected by β galactosidase staining. However, as β galactosidase is also a senescence marker, this result could reflect high neuronal senescence rather than low microglial C9orf72. Surprisingly, mouse models in which the *C9orf72* orthologue has been knocked down, generally do not display motor neuron pathology as expected. Instead, they have autoimmune-like conditions with microglial and macrophage dysfunction [110,111] together with increased inflammatory cytokines in serum [111] and spinal cord [110]. Furthermore, *C9orf72* knockdown microglia and macrophages appear to display lysosomal dysfunction, which may result in a reduced ability of microglia to clear cellular debris. Transcriptomic characterization of these microglia revealed a predominantly inflammatory phenotype, with increased expression of TNFα and IL-1β, in knockdown mice compared to controls [110]. Together, these results imply that the reduced levels of C9orf72 would be associated with an inflammatory microglial response and lysosomal dysfunction in macrophages, features commonly observed in MND models [112].

The presence of discrete microglial phenotypes

Older research (predominantly in transgenic mouse models with MND-causing *SOD1* mutations) has raised the possibility of two phases of microglial activation: an earlier phase prior to symptom onset in which microglia express more genes historically associated with anti-inflammatory processes, followed by a later phase associated more so with expression of pro-inflammatory factors with the disease progression [44,113–116].

However, transcriptional studies using the same model have had conflicting results, with microglia expressing molecules classically associated with both anti- and pro-inflammatory functions throughout disease. Flow cytometry and RNA sequencing of microglia from mSOD1^{G93A} mouse spinal cord over different disease time points have revealed simultaneous upregulation of pro-inflammatory genes such as *TNF-α*, *IL-1β*, and receptors for *IFNs*, and *NOX2* , as well anti-inflammatory genes such as *Insulin Growth Factor-1*, *Progranulin* and *DAP12* / *TYROBP* [45]. These microglia significantly differed in phenotype to those WT microglia stimulated with LPS, indicating a disease-specific profile.

Table 2. Summary of transcriptomic studies of microglia in mSOD1^{G93A} models

Type of model (treatments conditions)	*Method of microglial isolation*	*Location*	*Stage of disease*			*Method*	*References*
			Presymptomatic stage	*Symptom Onset – Early stage*	*Late/end stage*		
mSOD1^{G93A} mouse spinal cord homogenate	Not Applicable	lumbar spinal cord [L1-L5]	No change	↑Caspase-1, ↑TNF-α, ↑JAK3, ↑CD68, ↑Retinoid X receptor α, ↑Homeobox proteins,↑GABAA-R	↑↑Vimentin, ↑↑CD147, ↑Caspase-1, ↑↑Bcl-xL ↑↑Cathepsin-D, ↑↑TNF-α, ↑↑JAK3, ↑↑CD68, ↑GABAA-R	cDNA microarray, macroarray and RT-qPCR	[92]
mSOD1^{G93A} mouse microglia	Flow Cytometry (CD11b and CD16/CD32)	Lumbar Spinal Cord	Not measured	↑NOX2	↑↑NOX2, ↓BDNF, ↓Ym1, ↓↓CD163	RT-qPCR	[44]
mSOD1^{G93A} mouse microglia	Sequential Percoll gradient centrifugation and magnetic bead isolation. Antibodies conjugated to CD11b	Total spinal cord	↑MMP-12, ↑IGF, ↑Optineurin, ↑NOX2	↑↑IGF, ↑↑Optineurin, ↑↑NOX2, ↑TNF-α, ↑IL-1B, ↑TREM2, ↑Progranulin, ↑↑MMP-12	↑↑↑IGF, ↑↑↑Optineurin, ↑↑↑NOX2, ↑↑MMP-12, ↑↑Progranulin, ↑↑TNF-α, ↑↑IL-1B, ↑IL-α ↑TREM2, ↑DAP12,↑IL-10, ↑ApoE, ↑Tau, ↑Osteopontin	Microarray and RNAseq [over 1000 genes were differentially expressed therefore only given a sample)	[45]
mSOD1^{G93A} Rat microglia	Magnetic Bead Column Purification Antibodies Conjugated to CD11b/c	Cortex	No change			RT-qPCR	[28]
		Brain Stem	No change	↑Osteopontin, ↑Galectin	↓TNF-α, ↓IL-6, ↑↑Osteopontin, ↓IL-10, ↑VEGF, ↑↑Galectin		
		Spinal cord: Cervical [C1-C7]	↑MCP-1	↓TNF-α, ↑MCP-1,↑Osteopontin, ↑VEGF,↑Galectin-3	↓IL-6, ↓↓TNF-α, ↑MCP-1,↑↑Osteopontin, ↓Arg1, ↑↑VEGF,↑↑Galectin		
		Spinal Cord: Thoracic [T2-10]	No change	↓TNF-α,↑Osteopontin, ↑Galectin, ↑VEGF	↓IL-6,↓↓TNF-α,↑↑Osteopontin, ↓↓↓Arg1,↑↑Galectin, ↑↑VEGF		
		Spinal Cord: Lumbar [L1-L5]	No change	↓TNF-α,↑Osteopontin, ↓IL-10, ↑VEGF,↑Galectin	↓↓TNF-α, ↓IL-6, ↑↑Osteopontin,↓↓Arg1, ↓↓IL-10, ↓BDNF, ↑↑VEGF, ↑↑Galectin		
mSOD1^{G93A} mouse microglia cultures (LPS Treated Adult microglia)	Not Given - States microglia were isolated, purity checked using CD11b immunostaining	Whole brain	(Foetal Microglia) No change	↑TNF-α,↓IL-6	Not measured	Enzyme-Linked Immunosorbent Assay (ELISA)	[96]

Key: Inflammatory, Anti-inflammatory, and Other. ***Note:*** Arrows represent change in expression since last measured point and do not reflect the magnitude of change. All changes compared to control littermates apart from Nikodemova *et al.*[28], which compared to wild-type mice.

In comparison, RT-qPCR characterization of microglia from mSOD1^{G93A} rat spinal cord did not find microglial activation in the presymptomatic stages of disease [28]. However, at disease onset and late-stage disease, mSOD1 microglia showed a simultaneous upregulation of *galectin* and *osteopontin*, factors involved in macrophage activation and chemotaxis, which have been observed as being both neurotoxic and trophic, and *Vascular Endothelial Growth Factor (VEGF)* a pro-survival factor. This was accompanied by the concomitant downregulation of the classic pro-inflammatory marker *TNF-α*, and anti-inflammatory/ trophic markers *BDNF*, *IL-6* and *arginase-1*.

The same study showed microglial function to vary according to location [28]: microglia from the cortex, brain stem and the cervical, thoracic and lumbar spinal cord regions were compared. The above differences were only seen in the spinal cord, but not in cortical regions. However, the cortical region where microglia were sampled was not described. It is thus possible that either unaffected cortices (such as occipital cortex) rather than the motor cortex were sampled, or total brain which may 'dilute' subtle changes. These differences in microglial transcriptome indicate that microglial function may vary with both anatomical region and disease stage. This and other studies [49,50] establish that the inflammatory environment, and the inflammatory response to disease differs between regions. It is thus important that the anatomical location is clearly defined when performing such studies in MND. It is also useful to consider more than one CNS region in such research.

The most relevant mSOD1 murine studies of microglial transcriptome have been summarized in Table 2, highlighting the discrepancies in results. It is of interest to note, even studies comparing the same regions of spinal cord in mSOD1 mice at the same stage of disease have shown differing results.

The inconsistency in these results likely reflects a number of differences in the methodology, including varying methods of gene quantification and the sample isolation protocol e.g. isolated microglia versus tissue homogenate versus cultured microglia. Microglial phenotype is highly dependent on their environment, therefore removing these cells from their *in vivo* environment will alter their transcriptional profile. Furthermore, microglia cell lines and cultures have been found to be both functionally and transcriptionally different to *ex vivo* microglia [117,118] .

Variability in the genetic background could also cause variation in the results. In the mSOD1^{G93A} mouse model, four copies of the transgene, which results in mSOD1 expression equivalent to those expression levels seen in human MND, is insufficient to produce symptoms in mice [74]. However, increasing the transgene copy number not only results in earlier symptom onset but also produces a more rapid disease progression [74,119]. The genetic background of the mice used for breeding has also been found to alter the life span and rate of disease progression, despite containing the same mutation and expressing the same quantity of mutated protein [120–122]. This potentially reflects the variability observed in humans, with patients carrying G93C *SOD1* mutations varying in survival time from approximately 5 to 20 years [123]. Moreover, the sex of the mice appears to affect the rate of disease progression with various reports indicating increased survival time and delayed symptom onset in female mice compared to males [121,124,125], as well as sex differences in response to treatment [125–127].

Microglia as a therapeutic target

With their clear involvement in MND, microglia have been a target in the development of therapeutic options; the predominant aim being to reduce inflammation[34]. The administration of anti-inflammatory drugs has had mixed results in murine models and has not translated well to humans. Minocycline, a tetracycline antibiotic with broad spectrum anti-inflammatory activity, has been found to cross the blood-brain barrier, and reduce activity of inflammatory and pro-apoptotic enzymes [128]. In early trials, administration of minocycline was found to delay symptom onset and improve survival in mSOD1^{G93A} and mSOD1^{G37R} mice, if administered before symptom onset [129–131]. However, administration after disease onset accelerates disease progression and increases microgliosis [132,133], supporting a beneficial role for microglia in the earliest stage of the disease. In human trials, minocycline was found to increase the rate of decline, significantly reducing lifespan [134]. Similarly, trials of ceftriaxone (antibiotic), celecoxib (anti-inflammatory),

and thalidomide (immunomodulatory drug) showed promise in mSOD1 mice, but were ineffective in humans [135,136]. Criticisms about the original animal trials have been raised including whether adequate sample powering was employed, unaccounted confounding variables and the efficacy of administering a drug before disease onset[137].

Another drug Clemastine, which reduces inflammation while increasing the expression of the anti-inflammatory cytokine arginase-1, has also failed to improve disease in mSOD1^{G93A} mice, unless administered within the presymptomatic phase [138,139].

While attempts to dampen neuroinflammation have given mixed results, targeting specific inflammatory pathways has shown greater success. Inhibition of the previously described NF-κB pathway *in vitro* in mSOD1^{G93A} microglia rescued motor neuron axon length and survival time to levels seen with wild-type microglia [105]. This was accompanied by reduced expression of TNF-α and nitric oxide in mSOD1^{G93A} microglia, to levels observed in wild-type microglia [105]. Transgenic NF-κB inhibition in mSOD1^{G93A} mice resulted in a 47% increase in life expectancy compared to nontreated mSOD1^{G93A} mice [105], emphasizing the effectiveness of targeting this pathway. Similar manipulations have not yet been tried in humans.

Furthermore, there has been some success utilizing treatments which aim to mimic the supportive microglial functions in mSOD1 mouse models. The administration of the trophic factors, VEGF and IGF-1, at disease onset using viral vectors, has improved the motor symptoms and extended survival time [140,141].

Microglia show potential as a therapeutic target in MND treatment. However, it is unsurprising that to date, attempts in humans have been unsuccessful given the conflicting findings observed in animal models of MND, and our limited understanding of which microglial functions are relevant to human MND.

Future directions

Research using animal models has shown that microglia contribute to MND pathology with both toxic and trophic functions. However, attempts to subclassify microglial functions, based on the compounds expressed by these cells, have been inconsistent. Therefore, more work is required using human *post mortem* CNS tissue to characterize the microglial phenotype over the course of the disease.

Despite the inherent challenges that come with using *post mortem* tissue, it has many benefits over mSOD1 mouse models of MND. These models significantly differ from human sporadic MND pathology. Transgenic mSOD1 models greatly overexpress mSOD1 resulting in a severe phenotype that progresses much faster than human MND [79] and could mask more subtle pathological changes. Furthermore, mutations in *SOD1* are observed in only ~2.5% of all MND cases [4], and represent a very specific form of MND. Indeed, *SOD1* fMND cases do not develop the hallmark ubiquitylated TDP-43^{+} inclusions seen in sporadic human MND and most forms of fMND, including *C9orf72, Angiogenin, Ataxin 2,* and *TANK Binding Kinase 1* cases. Consequentially, mSOD1 models may not be representative of the majority of human MND. Models have been developed using other familial mutations, such as *C9orf72, PFN1* and *TARDBP* [79]. Some of these models do show characteristic TDP-43 pathology. However, they also rely on high transgene copy numbers to produce a phenotype, and in some models (namely models utilizing mutations in *C9orf72*) the disease phenotype can be unpredictable, with a rapid disease progression and a survival time of less than 6 months [79].

Furthermore, transcriptional single-cell sorting of immune cells from the human cortex has demonstrated several uncharacterized microglial phenotypes, with some of them unique to humans [47]. This further supports the importance of transcriptional profiling in humans as opposed to experimental models. Characterization of microglia isolated from different regions of human brain and spinal cord using multiplexed single-cell mass spectrometry has confirmed the presence of distinct microglial populations in different locations [48], in a similar manner to observations made in mSOD1 murine models [28]. Therefore, it is important to examine microglial transcripts separately across regions of the brain and spinal cord, to better characterize the different populations of microglia. It may also be essential to characterize microglia in seemingly unaffected areas, such as occipital cortex to determine if there are microglial functions, which make these regions less susceptible to MND pathology.

In summary, microglial activation is a clear feature of MND; both in humans and mouse models, and thus

modulation of the microglial phenotype in MND could help to promote neuronal survival. However, we need first to determine and identify which microglial functions are important to human disease with TDP-43 proteinopathy.

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Data availability statement

Data sharing is not applicable to this article as no new data were created or analysed in this study.

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