**Title: Microglia in Motor Neuron Disease**

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**Key Words:** Motor Neuron Disease, microglia, inflammation, humans,

**Word Count:** 5681

**Abstract Word Count:** 185

**Figures:**

**Figure 1.** Microglial Morphology

**Figure 2.** The Cycle of Microglial/ Neuronal Neurotoxicity

**Table 1.** Summarisation of Microglial Phenotypes, their activating stimuli and typical markers for their identification.

**Table 2**. Summary of Transcriptomic Studies of Microglia in mSOD1G93A Models

**Acknowledgments:**

The authors would like to thank the Pathological Society of Great Britain for funding this work

as PhD studentship which supports the first author.

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

**Abbreviations:**

|  |  |
| --- | --- |
| Motor Neuron Disease | MND |
| Amyotrophic Lateral Sclerosis | ALS |
| sporadic Motor Neuron Disease | sMND |
| familial Motor Neuron Disease | fMND |
| mutant SOD1 | mSOD1 |
| Pathogen-Associated Molecular Patterns | PAMPs |
| Damage-Associated Molecular Patterns | DAMPs |
| Positron Emission Tomography | PET |
| 18-kDA Translocator Protein | TSPO |
| Cerebrospinal Fluid | CSF |
| Lipopolysaccharide | LPS |
| mutant TDP-43 | mTDP-43 |
| Enzyme-Linked Immunosorbent Assay | ELISA |

# Abstract

Motor Neuron Disease (MND) is a fatal neurodegenerative condition, which is characterised 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, are 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 characterised the microglial response to MND, these studies have painted a complex and often contradictory picture, indicating a need for further characterisation 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.

# Motor Neuron Disease

Motor Neuron Disease (MND) is a fatal neurodegenerative condition characterised by the progressive degeneration of both upper motor neurons in the motor cortex, and the lower motor neurons in the brain stem and spinal cord1. MND has a number of subtypes, of which Amyotrophic Lateral Sclerosis (ALS) is the most common and affects around 2 in every 100,000 people2. 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 fatigue1. Diagnosis of MND normally occurs around 55-60 years of age. While survival time varies from just a few months to decades3, 80% of patients survive only two to five years after diagnosis, with death usually resulting from paralysis and respiratory failure1. 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 months1.

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 inheritance4. More than forty pathogenic mutations have been associated with MND, including mutations in *SOD1* (*Superoxide dismutase*)5, *TARDBP* which encodesTransactive Response DNA-binding protein 43 kD (TDP-43)6*, FUS* (*RNA Binding Protein Fused in Sarcoma/Translocated in Sarcoma)*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 neurons15.

The hallmark microscopic pathology of MND are the presence of protein aggregates in the remaining motor neurons. In the majority of cases, these aggregates are composed of ubiquitylated TDP-436. However, aggregates may also include or be composed of other proteins including FUS and SOD116.TDP-43-positive inclusions are primarily seen in the motor structures of the spinal cord, corticospinal tract and the motor cortex17. These inclusions are also observed to a lesser and more variable extent in extra-motor regions including the anterior frontal and temporal cortices18. MND is considered to be on a disease spectrum with Frontotemporal Dementia which is characterised by changes in personality and behaviour, loss of language skills and cognitive decline19. Along this continuum cases show pathology purely in motor regions, ranging to FTD cases with purely non-motor region pathology18. 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.

# 1.2 Glial Involvement in MND

In models of MND, motor neurons show dysfunction in many cellular processes including axonal transport and microtubule function20,21, RNA processing22, endoplasmic reticular stress response23, and endosomal trafficking24. 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 models25,26. Microgliosis and astrogliosis, the emergence of activated microglia and astrocytes respectively, can be seen in human *post-mortem* tissue and animal models27,28. Further, in a chimeric mutant SOD1G93A 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 glia29, 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 MND27,30,31, 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 immunity32.

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 microenvironment33. 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 receptors34. 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 aging and chronic activation microglia have been observed to adopt another phenotype, often referred to as the dystrophic phenotype 35,36 . These cells show reduced surveillance and dendritic branching37, a redistribution of Iba1- particularly in the processes38, and reduced motility and migration39. Furthermore, these cells show changes in gene expression including expression of pro-inflammatory cytokines in homeostatic conditions40 and more sustained responses to damage39, as well as storage of iron as ferrate41. As this phenotype is most commonly observed in the aging 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 injury32. 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 phagocytosis42.

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 activated microglia may fall on a spectrum, with some cells being purely M1 through to some cells which show a purely M2 phenotype43,44. 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 profiles45,46. 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 phenotypes47,48. This said, a lot of the literature still follows this type of characterisation 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 CNS49,50, microglial age51, activating stimuli and disease47, as well as well as differences between human and murine microglia51. 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 has 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 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 death52. This process is seen as a driver of progression in neurodegeneration52,53.

# 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 microglia31. 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 above31,54,55.

A population of microglia, which are immunopositive for the haemopoetic transmembrane phosphoglycoprotein, CD34, have been observed in the ventral horn of sMND spinal cord56. 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 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 control54. This has also been observed to a lesser extent in non-motor regions including the amygdala, hippocampus, and the middle frontal and superior temporal gyri54. 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 function54.

Additionally, increased microglial activation, as illustrated by CD68 and Iba1 immunostaining, in the corticospinal tract has been significantly associated with more rapid disease progression55. Familial cases associated with *C9orf72* mutations tend to show more extensive microglial pathology within the corticospinal tract55. 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 ligand61. 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 extra-motor 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 Scale62. 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 cytokines63. Soluble TREM2, a cleaved form of the protein, was found to be upregulated in human MND patient spinal cord and associated with improved patient survival64,65. 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 disease65. In the late stages of disease, *TREM2* expression correlated with survival time in MND patients, perhaps reflecting a continued neuroprotective response65. 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 varied64,67.

A recent study by Zhou *et al.*68 has found *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 found TREM2 was overwhelmingly expressed by infiltrating monocytes, with little expression was observed in native microglia69. 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-γ70–72. Furthermore, microarray transcriptomic analysis of human spinal cord grey matter has found an increase in inflammatory signalling in MND, characterised by various interleukins and cathepsins73.

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 favour 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 utilised transgenic animal models. The first made, and most widely utilised and characterised MND model was generated in mice using the familial G93A mutation in the *SOD1* gene74. Further mSOD1 models have been developed using other patient variants75–77. The mSOD1 models rely on high transgene copy numbers, which produces a widespread over expression of mutant protein, resulting in a reliable but severe disease phenotype78. 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 span79.

Following this, models have been created which contain transgenic mutations in *TARDBP, FUS, C9orf72, PFN1* and *UBQLN2* (summarised by Lutz79), as well as in other species including rats80 and zebrafish81–83. The MND models replicate many of the keys 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 features78.

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## 4.1 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 respectively84. In contrast, decreased levels of Iba1+ microglia in mSOD1G93Amice spinal cord, prior to symptom onset have also been reported85. Furthermore, no change in microglial number was observed in mSOD1G93A rat spinal cord isolated at the equivalent pre-symptomatic stage28. However, a significant increase in the number of microglia was observed immediately post-symptom onset28. 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 characterise 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.

## 4.2 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 pathogens86. 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 mSOD1G93A mice has identified high levels of ***Ym1*,** anti-inflammatory macrophage marker as well as Brain-derived Neurotrophic Factor (BDNF), a neurotrophin which promotes neuronal survival44. 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 barrier89, indicating this process could occur early in disease. Co-culture 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 pre-symptomatic mSOD1G93A mice has demonstrated a 16-fold increase in IL-10 production, a cytokine responsible for inflammatory cytokine downregulation90. 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 production91.

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

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## 4.3 Pro-inflammatory Microglia in animal models of MND

Transcriptional analysis of 11-week-old mSOD1G93A mouse spinal cord homogenates has detected a significant upregulation of inflammatory genes including cathepsin D and TNF-α92. This was thought to represent an inflammatory response in the pre-symptomatic stage. However, symptom onset has been observed as early as 8.5 weeks of age in this model85, implying the inflammatory microglial phenotype observed actually occurs in the early, post-symptom-onset stage of disease. Furthermore, these results contradict other findings of increased anti-inflammatory cytokine expression at the same age in the same mSOD1G93A model44.

The latter study went on to examine late stage mSOD1G93A mice and found a shift towards diminished anti-inflammatory markers and high levels of nitric oxide, a toxic immune mediator. Co-culture of these late stage microglia with wild-type motor neurons induced neuronal death44, likely by increasing motor neuron sensitivity to glutamate, potentially resulting in excitotoxicity93, a known feature of motor neuron pathobiology in MND. These microglia also appear to inhibit the protective uptake of glutamate by astrocytes in culture93.

Microglia expressing mSOD1 appear to be more neurotoxic than wild-type microglia. Upon stimulation with the neurotoxicity-inducing PAMP, Lipopolysaccharide (LPS), microglia over-expressing mSOD1G93A released more NO and superoxidase, than both microglia overexpressing wild-type *SOD1*, and wild-type microglia94,95. These mSOD1 microglia induced more neuronal death in co-culture with motor neurons than their wild-type counterparts.

Interestingly, Weydt and colleagues96 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 mSOD1G93A microglia showed a significant difference from the wild-type control, with increased *TNF-α* production and decreased *IL-6* expression. This suggests adult and not perinatalmSOD1-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 mSOD197. Microglial priming is a feature of aging and other neurodegenerative conditions such as Alzheimer’s disease98: these microglia are less responsive to anti-inflammatory agents, promoting chronic inflammation98.

Furthermore, transplantation of wild-type microglia into mSOD1G93A mice, via bone marrow transplant, results in reduced MN loss and extended survival95, 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 activation99. The V249I variant of *CX3CR1* is associated with faster progression and shorter survival in human sMND100. Transgenic mSOD1G93A 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.

## 4.4 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 microglia102. 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 co-cultured with primary mSOD1G93A mouse motor neurons, these primed microglia proved to be neurotoxic, compared to un-primed mSOD1G93A microglia. However, incubation of mSOD1 aggregates with motor neurons alone had no significant effect on motor neuron survival, indicating 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 characterised by *TNF-α, IL-1β* and *NOX2* in primary mouse microglia, compared with wild-type treated with TDP-43 or non-treatment103. 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) pathway103.The NF-κB pathway is a master regulator of inflammation, and is highly upregulated in MND patient spinal cord, and mSOD1 mice104,105. When activated, the NF-κB complex relocates to the nucleus, inducing further pro-inflammatory cytokine and chemokine expression106. 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 microglia103. This results in the production of pro-inflammatory cytokines103,105. 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 microglia105.

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 dysfunctional microglia may contribute to the pathological translocation of TDP-43 from the nucleus to the cytoplasm. Using a transgenic zebrafish model, which co-expresses green fluorescent protein-tagged TDP-43 and microglia which express mCherry, *Svahn et al.*107 visualised 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 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 mice108,109. In contrast, another study suggested 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 dysfunction110,111 together with increased inflammatory cytokines in serum111 and spinal cord110. 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 characterisation of these microglia revealed a predominantly inflammatory phenotype, with increased expression of TNFα and IL-1β, in knockdown mice compared to controls110. 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 models112.

## 4.5 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 progression44,113–116.

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 mSOD1G93A 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.

In comparison, RT-qPCR characterisation of microglia from mSOD1G93A rat spinal cord did not find microglial activation in the pre-symptomatic stages of disease28. 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 found microglial function to vary according to location28: 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 microglial function may vary with both anatomical region and disease stage. This and other studies49,50 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 summarised 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 found 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, 4 copies of the transgene, which results in mSOD1 expression equivalent to those expression levels seen in human MND, is insufficient to produce symptoms in mice74. However, increasing the transgene copy number not only results in earlier symptom onset but also produces a more rapid disease progression74,119. 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 protein120–122. This potentially reflects the variability observed in humans, with patients carrying G93C *SOD1* mutations varying in survival time from approximately 5 to 20 years123. 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 males121,124,125, as well as sex differences in response to treatment125–127.

# 5. 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 inflammation34. 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 enzymes128. In early trials, administration of minocycline was found to delay symptom onset and improves survival in mSOD1G93A and mSOD1G37R mice, if administered before symptom onset129–131. However, administration after disease onset, accelerates disease progression and increases microgliosis132,133, 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 lifespan134. Similarly, trials of ceftriaxone (antibiotic), celecoxib (anti-inflammatory), and thalidomide (immunomodulatory drug) showed promise in mSOD1 mice, but were ineffective in humans135,136. 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 onset137.

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 pre-symptomatic phase138,139.

While attempts to dampen neuroinflammation have given mixed results, targeting specific inflammatory pathways have 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 microglia105. This was accompanied by reduced expression of TNF-α and nitric oxide in mSOD1G93A microglia, to levels observed in wild-type microglia105. Transgenic NF-κB inhibition in mSOD1G93A mice resulted in a 47% increase in life expectancy compared to non-treated mSOD1G93A mice105, emphasizing the effectiveness of targeting this pathway. Similar manipulations have not yet been tried in humans.

Furthermore, there has been some success utilising 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, have improved the motor symptoms and extended survival time140,141.

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.

# 6. Future Directions

Research using animal models has shown microglia contribute to MND pathology with both toxic and trophic functions. However, attempts to sub-classify 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 characterise the microglial phenotype over the course of the disease.

Despite in the inherit 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 over express mSOD1 resulting in a severe phenotype that progresses much faster than human MND79 and could mask more subtle pathological changes. Furthermore, mutations in *SOD1* are observed in only ~2.5% of all MND cases4, 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 utilising mutations in *C9orf72*) the disease phenotype can be unpredictable, with a rapid disease progression and a survival time of less than 6 months79.

Furthermore, transcriptional single cell sorting of immune cells from the human cortex has demonstrated several uncharacterised microglial phenotypes, with some of them unique to humans47. This further supports the importance of transcriptional profiling in humans as opposed to experimental models. Characterisation 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 locations48, in a similar manner to observations made in mSOD1 murine models28. Therefore, it is important to examine microglial transcripts separately across regions of the brain and spinal cord, to better characterise the different populations of microglia. It may also be essential to characterise 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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