

## COMMENTARY

## Thoughts &amp; Opinion

# Recent insights from human induced pluripotent stem cell models into the role of microglia in amyotrophic lateral sclerosis

Lara M. Nikel<sup>1,2</sup> | Kevin Talbot<sup>1,2</sup>  | Björn F. Vahsen<sup>1,2</sup> 

<sup>1</sup>Oxford Motor Neuron Disease Centre, Nuffield Department of Clinical Neurosciences, John Radcliffe Hospital, University of Oxford, Oxford, UK

<sup>2</sup>Dorothy Crowfoot Hodgkin Building, Kavli Institute for Nanoscience Discovery, University of Oxford, Oxford, UK

## Correspondence

Kevin Talbot and Björn F. Vahsen, Oxford Motor Neuron Disease Centre, Nuffield Department of Clinical Neurosciences, John Radcliffe Hospital, University of Oxford, Oxford OX3 9DU, UK.

Email: [kevin.talbot@ndcn.ox.ac.uk](mailto:kevin.talbot@ndcn.ox.ac.uk) and [bjorn.vahsen@ndcn.ox.ac.uk](mailto:bjorn.vahsen@ndcn.ox.ac.uk)

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## Abstract

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease, primarily leading to the degeneration of motor neurons. The traditional focus on motor neuron-centric mechanisms has recently shifted towards understanding the contribution of non-neuronal cells, such as microglia, in ALS pathophysiology. Advances in induced pluripotent stem cell (iPSC) technology have enabled the generation of iPSC-derived microglia monocultures and co-cultures to investigate their role in ALS pathogenesis. Here, we briefly review the insights gained from these studies into the role of microglia in ALS. While iPSC-derived microglia monocultures have revealed intrinsic cellular dysfunction due to ALS-associated mutations, microglia-motor neuron co-culture studies have demonstrated neurotoxic effects of mutant microglia on motor neurons. Based on these findings, we briefly discuss currently unresolved questions and how they could be addressed in future studies. iPSC models hold promise for uncovering disease-relevant pathways in ALS and identifying potential therapeutic targets.

## INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is characterized by a loss of motor neurons (MNs) in the brain and spinal cord, leading to progressive paralysis and death, usually by respiratory failure. ALS exhibits considerable genetic heterogeneity, with 12%–15% of patients having a single disease-determining genetic mutation, in both familial cases, but also in apparently “sporadic” cases. A hexanucleotide repeat expansion (HRE) in the *C9orf72* gene occurs in ~40% of familial cases, with 20% affected by mutations in *SOD1*, and much less frequent mutations in *TARDBP* (encoding the TDP-43 protein), *FUS*, and others.<sup>[1]</sup> 97% of ALS cases show nuclear clearing and cytoplasmic TDP-43 protein aggregation as the neuropathological hallmark. There is notable overlap with the related condition frontotemporal dementia (FTD), with

50% of FTD cases demonstrating TDP-43 proteinopathy. These disorders also share common genetic origins, notably involving *C9orf72* mutations.

The field's limited therapeutic success may in part be due to its longstanding MN-centric research focus. ALS research has recently shifted focus to include the contribution of non-neuronal cells.<sup>[1]</sup> Microglia, the immune cells of the central nervous system (CNS), play a crucial homeostatic role by continuously monitoring their surroundings and responding to injury and disease by changing their morphology, behavior, and gene expression. This activation can lead to a spectrum of pro-inflammatory and anti-inflammatory responses, depending on the specific environment. Some ALS-associated genes are highly expressed in microglia, including *C9orf72*, *FUS*, *PFN1*, *OPTN*, and *TBK1*, suggesting that mutations in these genes could have ALS-relevant effects on

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microglial biology.<sup>[1–3]</sup> Indeed, an activated microglial state has been described in animal models and human post-mortem studies, suggesting microglia have an early anti-inflammatory effect during in ALS, followed by a late proinflammatory phenotype (reviewed in more detail in ref.[1]). However, the relative effect of ALS-associated gene variants in promoting pro-inflammatory pathways over neurotoxicity as a secondary response to neuronal damage is still unclear.

Significant differences in the transcriptomic profiles of murine and human microglia impact the translatability of animal models to study microglia involvement in human disease. Induced pluripotent stem cells (iPSC) models enable the direct investigation of the effects of disease-relevant mutations in human cell types of interest. iPSC-derived MNs have yielded important insights into ALS pathophysiology, resulting in several clinical trials of therapeutic candidates for ALS.<sup>[4]</sup> More recently, iPSC models have been used to study molecular mechanisms in human microglia carrying ALS-associated genetic mutations. This commentary summarizes the recent advances in our understanding of microglial contributions to ALS using iPSC models and provides a perspective on future research.

## ALS-ASSOCIATED MUTATIONS ALTER MICROGLIAL BIOLOGY

iPSC-derived microglia monocultures offer a valuable platform for studying how ALS-associated mutations affect microglial function and identifying dysregulated mechanisms contributing to the disease (Figure 1A). Recent studies have investigated how ALS-associated mutations in *C9orf72*, *FUS*, and *PFN-1* impact microglial biology.

### *C9orf72* mutations

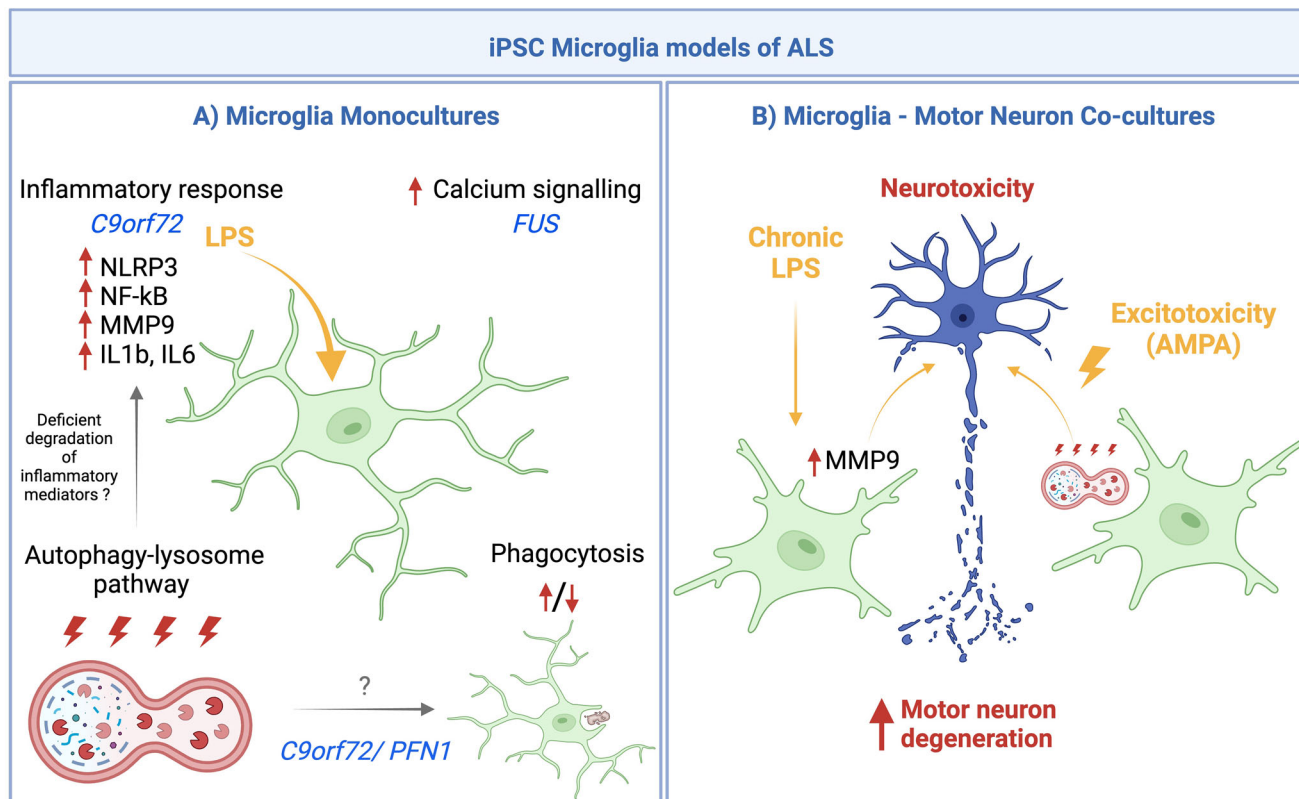
*C9orf72* has higher expression in iPSC-derived microglia compared to MNs.<sup>[5,6]</sup> We and others have investigated the intrinsic effects of the *C9orf72* HRE on iPSC-derived microglia cells in monoculture.<sup>[5–7]</sup> Interestingly, neither our study<sup>[5]</sup> nor Lorenzini et al.<sup>[7]</sup> found substantial differences in unstimulated *C9orf72* mutant microglia compared to controls by RNA sequencing, and baseline key inflammatory marker expression, such as *IL1B* or *IL6*, was unchanged.<sup>[5–7]</sup> These findings suggest that the mere presence of the *C9orf72* HRE does not inherently skew microglia towards a pro-inflammatory phenotype compared to control cells.

Interestingly, *C9orf72* mutant microglia demonstrated a heightened proinflammatory response to lipopolysaccharides (LPS), a well-defined inducer of a pro-inflammatory microglial phenotype, resulting in increased expression and release of pro-inflammatory mediators such as *MMP9*,<sup>[5]</sup> *IL1B*,<sup>[6]</sup> and *IL6*<sup>[6]</sup> and *NLRP3* and *NF-kB* activation.<sup>[6]</sup> In one of the studies, only a non-significant trend towards increased inflammatory cytokine expression in *C9orf72* mutant microglia was found, possibly because the shorter duration of LPS treatment (6 h) used<sup>[7]</sup> may have been insufficient to reveal a significant difference compared to the longer treatment durations used in the other

studies (8 h plus 12 h washout<sup>[6]</sup> and 48 h<sup>[5]</sup>). Of note, LPS is a useful tool for studying neuroinflammation, modeling the pro-inflammatory microglial response to endogenous stimuli such as neuronal death/damage, disease proteins (e.g., *TDP-43*), or cytokines released by microglia and other cell types during the disease process, but its bacterial origin may limit its translational relevance. Exploring more physiologically relevant stimuli, such as cytokines and damaged neurons, could enhance the translational relevance of experimental models for the microglial response in ALS. Altogether, the combined evidence indicates that the *C9orf72* HRE increases the responsiveness of *C9orf72* mutant microglia to external stimuli, and that an inflamed microenvironment is required to induce robust phenotypic changes.

But what is the cellular basis for this increased microglial response to pro-inflammatory stimulation? We have found that *C9orf72* expression is upregulated after 48h-treatment with LPS, both at gene and protein level,<sup>[5]</sup> implicating it directly in the microglial response to pro-inflammatory stimulation, although the exact mechanism remains unknown. There is evidence suggesting that *C9orf72* operates in cytokine degradation upon stress-removal. *C9orf72*'s involvement in autophagy and the endo-lysosomal system is well-established, and Banerjee et al.<sup>[6]</sup> demonstrated disrupted autophagy in *C9orf72* mutant microglia. Interestingly, their study has also shown decreased signal of pH-sensitive pHrodo-labeled bioparticles in *C9orf72* mutant microglia, which fluoresce in response to the acidic environment of endosomes and lysosomes upon phagocytic uptake. While the authors interpreted this as a phagocytic deficit,<sup>[6]</sup> it is equally plausible that this phenotype reflects impaired lysosomal acidification or degradation rather than an actual difference in the phagocytic capacity. Notably, Lorenzini et al.<sup>[7]</sup> found no conclusive evidence for deficient phagocytosis in *C9orf72* mutant microglia. The *C9orf72* HRE mutation therefore leads to a pro-inflammatory microglial phenotype, with provisional evidence for deficient degradation of inflammatory mediators through the autophagy-lysosome system upon prolonged stimulation or stress removal.

How does the *C9orf72* HRE mutation induce this molecular phenotype? There is now consensus that all three pathophysiological consequences of the *C9orf72* HRE (haploinsufficiency-mediated loss-of-function and gain-of-function toxicity through RNA foci formation and dipeptide repeat protein (DPR) synthesis) are present in *C9orf72* mutant microglia.<sup>[5–7]</sup> The high relative level of microglial *C9orf72* expression suggests that microglia might be vulnerable to loss-of-function. Indeed, homozygous *C9orf72* knock-out iPSC microglia replicated the heightened inflammatory response to LPS stimulation and autophagy deficit observed in *C9orf72* HRE microglia.<sup>[6]</sup> This suggests that reduced *C9orf72* expression disturbs the endo-lysosomal system, impairing inflammatory mediator clearance during prolonged inflammation. The biological effects of microglial gain-of-function toxicity remain unclear. Our study found only low DPR expression, which remained unchanged after LPS treatment, but noted substantial RNA foci formation (particularly anti-sense foci) in *C9orf72* mutant microglia.<sup>[5]</sup> It is unclear whether RNA foci might be upregulated in response to pro-inflammatory stimulation, but it is possible that



**FIGURE 1** (A) Summary of changes to microglial biology across different amyotrophic lateral sclerosis (ALS)-relevant mutations (*C9orf72*, *FUS*, *PFN1*) in iPSC microglia monocultures. Lipopolysaccharides (LPS) stimulation triggers an increased inflammatory response in mutant microglia (*C9orf72*<sup>[5,6]</sup>) which may be caused by deficient degradation by the autophagy-lysosome pathway. Impairments in autophagy and lysosomal degradation were found in *C9orf72* and *PFN1* mutant microglia.<sup>[3,6]</sup> There is evidence for changes in phagocytosis,<sup>[3,6]</sup> but it remains unclear whether phagocytosis is decreased or increased and whether this is a true effect or due to impairments in the autophagy-lysosome pathway. Increased calcium signaling was found in *FUS* mutant microglia.<sup>[2]</sup> (B) Non-cell autonomous neurotoxicity of *C9orf72* microglia (green) caused by an excitotoxic or chronic inflammatory insult as shown in microglia-MN co-cultures. MN (blue) degeneration is mediated via microglial MMP9 release<sup>[5]</sup> and impaired microglial autophagy<sup>[6]</sup> following chronic LPS stimulation or AMPA-induced excitotoxicity, respectively. Figure created using BioRender.com.

gain-of-function mechanisms further contribute to the pro-inflammatory microglial phenotype under chronic inflammatory conditions.

## FUS mutations

Kerk et al.<sup>[2]</sup> showed significant transcriptomic changes in engineered homozygous P525L *FUS* mutant microglia, including upregulation of chemoreceptor gene expression associated with increased calcium flux upon ligand binding to two chemoreceptors (P2RY6, GPR183) in homozygous, and to a lesser extent, heterozygous *FUS* mutant microglia. Despite transcriptomic alterations in microglial function-related genes, neither phagocytosis nor cytokine release differed between mutant and healthy microglia, possibly because alternative stimuli to LPS were used (apoptotic MNs and myelin) which may not have adequately provoked an inflammatory response. The precise disease-relevant mechanism of the *FUS* P525L mutation in microglia remains unclear, though mislocalization of the *FUS* protein, a charac-

teristic feature of this mutation, was observed, which may contribute to the mutation's impact on microglial biology.

## PFN1 mutations

Introducing ALS-associated mutations (C71G, M114T) in profilin-1 (*PFN1*) to iPSC microglia caused significant proteomic and transcriptomic dysregulation associated with microglial lipid metabolism and vesicular degradation.<sup>[3]</sup> Interestingly, LPS stimulation produced no difference in cytokine release of IL6, IL10, CCL5, and TNF- $\alpha$  between *PFN1* mutant and wildtype microglia after 6 and 24-h, suggesting *PFN1* is not involved in microglial inflammation. Instead, *PFN1* mutants exhibited increased pHrodo bioparticle signal upon phagocytic uptake. This signal was caused by impaired vesicular degradation rather than enhanced phagocytosis, as determined by a phagocytosis washout paradigm. This was attributed to impaired vesicular processing through the endo-lysosomal pathway, affecting phagocytosis and autophagy. Both phenotypes were reversed by pharmacological autophagy

activation using rapamycin. Mechanistically, a gain-of-function effect of the mutation was proposed, as mutant PFN1 showed heightened binding to the signaling molecule PI3P involved in vesicular maturation and degradation. Notably, there is substantial overlap with the findings of Banerjee et al.,<sup>[6]</sup> with both studies suggesting impaired autophagy as a common phenotype in *C9orf72* and *PFN1* mutant microglia. Exploring the impact of mutations in other genes related to autophagy dysfunction, such as *OPTN* and *TBK1*, in microglia could offer further insights into the link between autophagy, microglia, and ALS. The absence of an inflammatory phenotype despite impaired vesicular degradation in *PFN1* mutant microglia requires further exploration, for instance, using a more chronic LPS paradigm<sup>[5]</sup> or washout after LPS treatment.<sup>[6]</sup>

### Microglia with ALS-associated mutations cause secondary neurotoxicity in co-culture models

Microglia are known to constantly survey their environment and closely interact with neurons and other glia cells. To understand their behavior in health and disease, their interactions with other cell types must therefore be considered. Multicellular co-culture systems such as the iPSC-derived MN-microglia co-culture developed by our lab,<sup>[8]</sup> are particularly valuable in investigating how ALS-associated microglial dysfunction is mechanistically connected with MN degeneration. Further, co-cultures support the maturation of microglia to a more ramified state than in monocultures, highlighting the advantage of modeling the brain environment using co-cultures.<sup>[4]</sup>

We,<sup>[5]</sup> and others,<sup>[6]</sup> have recently investigated direct toxic effects of *C9orf72* mutant microglia on MNs in co-culture (Figure 1B). Both studies found microglial neurotoxicity only in response to stimulation, consistent with the observation that pro-inflammatory microglial properties in monoculture are only triggered in inflammatory environments. Specifically, Banerjee et al.<sup>[6]</sup> demonstrated that mutant microglia exacerbated MN death in response to the excitotoxic stimulus AMPA. They attributed this non-cell autonomous toxicity to impaired microglial autophagy, as pharmacological autophagic activation using rapamycin mitigated the increased MN death and release of the pro-inflammatory mediator IL1B in co-culture. Complementing this finding, we showed that chronic LPS stimulation of *C9orf72* mutant microglia co-cultured with healthy MNs upregulated MMP9 expression and increased apoptotic signaling and MN death compared to co-cultures with control microglia.<sup>[5]</sup> Administering an MMP9 inhibitor ameliorated this non-cell-autonomous microglial toxicity, suggesting that MMP9 is one of the molecular mediators. Interestingly, we identified that additional growth factors, cytokines, and pro-inflammatory molecules were dysregulated in co-culture supernatants, several of which were not found in mutant microglia monocultures. It remains to be determined whether any of these are involved in microglial toxicity, but this further highlights the importance of cell-cell interactions for uncovering the microglial contribution to neurotoxicity in ALS. Both studies agree that *C9orf72* mutant microglia possess latent neurotoxicity, which becomes apparent in response to an excitotoxic insult

or a chronic pro-inflammatory stimulus, suggesting that microglial neurotoxicity in *C9orf72*-ALS is secondary to neuronal damage.

### Open questions and future directions

We now know that ALS-associated mutations impact microglial biology and can lead to secondary non-cell autonomous toxicity in *C9orf72*-ALS. However, it is unclear whether this toxicity is specific to the *C9orf72* HRE or consistent across different ALS-associated mutations and sporadic ALS. Integrating insights from multicellular models across diverse disease-relevant mutations and sporadic cases will enhance our understanding of the microglial contribution to ALS pathogenesis and potential treatment strategies. Specifically, it will be important to explore whether microglia contribute to TDP-43 proteinopathy, the key neuropathological hallmark in almost all ALS cases. Limited evidence suggests that TDP-43 may influence microglial function, extracellularly by promoting a pro-inflammatory state<sup>[1]</sup> and intracellularly by regulating microglial phagocytosis as shown in a TDP-43 knockout mouse model of Alzheimer's disease.<sup>[9]</sup> *TARDBP*-mutant microglia will be a useful initial tool in understanding the impact of both microglial and neuronal TDP-43 dysfunction on microglia and in revealing common pathways relevant to TDP-43 pathology.

More refined iPSC models are required to enhance the study of microglial involvement in ALS, including specific protocols to generate microglia of a regionally defined (e.g., spinal or cortical) identity to identify differential effects in the context of the same mutation. Astrocyte-microglia-MN tri-cultures and organoids, will illuminate the role of astrocytes in the inflammatory environment required to elicit mutation-specific changes in microglia and trigger MN damage in ALS. A recent study showed that conditioned astrocyte medium decreased calcium flux in co-cultures of *C9orf72* mutant MNs and microglia but not in MN monocultures, highlighting the importance of multicellular models.<sup>[10]</sup> Organoids contain a complex microenvironment of diverse cell types, offering insights into the contributions of various non-neuronal cells beyond microglia in ALS pathogenesis if methodological consistency in cell type proportions and complexity can be achieved. Notably, most organoids do not spontaneously develop microglia, necessitating the development of ALS-relevant protocols that incorporate separately differentiated microglia into cortical or spinal organoids.

### CONCLUSIONS

The exploration of microglia in ALS pathology through iPSC models suggests roles for both cell-autonomous and non-cell autonomous dysregulation in disease. While monoculture studies have delineated distinct molecular pathways affected by ALS-associated mutations, co-culture models have uncovered a microglial role in mediating neurotoxicity in response to exogenous stimuli. Moving forward, using iPSC models to dissect shared pathways across diverse ALS mutations and incorporating insights from multicellular models holds promise

for identifying novel therapeutic targets and advancing treatment strategies for this currently therapeutically intractable disease.

## AUTHOR CONTRIBUTIONS

Björn F. Vahsen conceived the idea. Lara M. Nikel and Björn F. Vahsen wrote the paper. Kevin Talbot edited the paper.

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## CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

## DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

## ORCID

Kevin Talbot  <https://orcid.org/0000-0001-5490-1697>

Björn F. Vahsen  <https://orcid.org/0000-0002-5159-5070>

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