Transcriptomic analysis of *C9orf72*-mutated iPSC-derived microglia implicates cell-autonomous upregulation of selected NLRP3 inflammasome genes in motor neuron degeneration

Running title: Dysregulated expression of inflammation and extracellular matrix genes in *C9orf72*-ALS microglia

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Data availability statement

Authors follow the rule of data transparency, and they have made sure that all data and materials, including software applications, support their published claims and comply with field standards.

Abstract

Hexanucleotide repeat expansion (HRE) in the non-coding region of the gene *C9orf72* is the most prevalent mutation in Amyotrophic Lateral Sclerosis (ALS) and frontotemporal dementia. The *C9orf72* HRE contributes to motor neuron degeneration in ALS both through cell-autonomous mechanisms and non-cell autonomous disease processes involving glial cells such as microglia. The molecular mechanisms by which *C9orf72* HRE microglia contribute to motor neuron death in ALS remain to be fully elucidated. In this study, we generated microglia from human *C9orf72* HRE and isogenic iPSCs using three different microglia derivation methods that can generate microglia with distinct intrinsic inflammatory profiles. RNA sequencing analysis reveals a consistent dysregulation of specific genes involved in pathways underlying NLRP3 inflammasome activation in *C9orf72* HRE microglia independent of priming with any activating cues. In agreement with elevated expression of NLRP3 inflammasome components, conditioned media from *C9orf72* HRE microglia enhance the death of *C9orf72* HRE motor neurons implicating microglia-secreted molecules in non-cell autonomous mechanisms of *C9orf72* HRE pathology. These findings provide evidence that *C9orf72* HRE microglia have a cell-autonomous pro-inflammatory phenotype that contributes to non-cell autonomous mechanisms of motor neuron degeneration in ALS through dysregulated expression of specific genes involved in NLRP3 inflammasome-mediated mechanisms.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is an incurable motor neuron disease characterized by the death of motor neurons in the cerebral cortex, brain stem, and spinal cord. ALS usually starts during adulthood and causes progressive paralysis leading to the death of most sufferers within 2 to 5 years following diagnosis, mainly due to respiratory failure. ALS also affects several non-motor systems and subcortical structures, and cognitive and behavioral impairments similar to frontotemporal dementia (FTD) symptoms are present in up to 50% of ALS patients (Benatar et al., 2024; Kirola et al., 2022).

The majority of ALS cases are sporadic, implying that the mechanisms triggering motor neuron loss are not exclusively inherited. However, approximately 10%-15% of ALS cases are transmitted in families: these cases are referred to as familial ALS. Multiple deleterious variants in numerous genes either drive motor neuron degeneration, increase susceptibility to the disease, or influence the rate of disease progression. Several ALS-mutated genes have a negative impact on shared cellular processes, including, but not limited to, RNA metabolism and protein homeostasis, nucleocytoplasmic trafficking, endoplasmic reticulum stress, dynamics of ribonucleoprotein bodies, mitochondrial functions, and autophagy (Goutman et al., 2022; Nijs and Van Damme, 2024).

Intronic hexanucleotide (GGGGCC – 'G_4C_2') repeat expansions (HRE) in the gene *chromosome 9 open reading frame 72* (*C9orf72*) are the most frequent genetic cause of ALS and FTD (DeJesus-Hernandez et al., 2011; Renton et al., 2011). In contrast to healthy individuals, who have, on average, 2-30 hexanucleotide repeats, ALS/FTD patients have *C9orf72* expansions with hundreds and even thousands of repeats. There are three (not mutually exclusive) proposed mechanisms to explain the effects of the *C9orf72* HRE: partial loss (haploinsufficiency) of *C9orf72* function caused by decreased expression of the *C9orf72*

mRNA and protein; toxic gain of function from the accumulation of sense G_4C_2 and antisense G_2C_4 repeat-containing RNA from bidirectionally transcribed expansions; and toxic gain of function from dipeptide repeat proteins aberrantly generated by repeat-associated non-ATG translation (Balendra and Isaacs, 2018; Gendron et al., 2018). The combination of loss- and gain-of-function mechanisms associated with the *C9orf72* HRE have wide-ranging consequences in multiple cell types, in agreement with the broad expression pattern of the *C9orf72* gene and the localization of the C9orf72 protein to multiple cellular compartments. Multiple cellular processes are affected in *C9orf72*-mutated motor neurons leading to altered mitochondria, oxidative stress, impaired autophagy, and impaired RNA metabolism, among other phenotypes (Chew et al., 2015; de Calbiac et al., 2024; Donnelly et al., 2013; Lopez-Gonzalez et al., 2016; Metha et al., 2021; Tran et al., 2015).

Like other neurodegenerative diseases, ALS is characterized by extensive neuroinflammation involving astrogliosis, activation of microglia, and infiltration of peripheral immune cells at sites of neuronal degeneration. Glial cells have a significant impact on disease onset and progression by mediating non-cell autonomous disease mechanisms (Appel et al., 2021; Calma et al., 2024; Izrael et al., 2020; Majewski et al., 2025). Intercellular communication between motor neurons and microglia, the resident immune cells of the central nervous system, play an important role in the degeneration of motor circuits in ALS. Microglia have the potential to perform both neuroprotective and neurotoxic roles in ALS, depending on their activation states in response to the local microenvironment. During initial phases of the disease microglia can protect motor neurons by performing scavenging, anti-inflammatory, and neuroprotective functions. During disease progression, however, with rising oxidative stress and injury within motor neurons, microglia can contribute to motor-neuron death by switching to pro-inflammatory and neurotoxic phenotypes (Beers and Appel, 2019; Majewski et al., 2025; Mimic et al., 2023). Much remains to be learned about the mechanisms underlying the dynamic

intercellular communication between motor neurons and microglia in ALS.

C9orf72 is robustly expressed in microglia and other myeloid-lineage cells. Analysis of post-mortem brains from *C9orf72*-ALS patients revealed morphological and immunological signs suggestive of microglia activation (Lant et al., 2014), as did *ex vivo* diffusion tensor MRI studies of tissues from ALS patients including *C9orf72* cases (Cardenas et al., 2017). Evidence that *C9orf72* is required for the normal function of myeloid cells came from studies in mice lacking the murine *C9orf72* ortholog in all tissues. *C9orf72*$^{-/-}$ (homozygous knockout) mice develop and age normally but exhibit several immune cell phenotypes, including lysosomal accumulation and altered immune responses in macrophages and microglia, with associated age-related neuroinflammation (Atanasio et al., 2016; Burberry et al., 2016; O'Rourke et al., 2016; Sudria-Lopez et al., 2016). Studies in which the human *C9orf72* HRE was introduced into mice showed signs of pathological expression of *C9orf72* HRE in the cortex including the appearance of hallmarks of microglia activation (Nicholson et al., 2018; Zhang et al., 2018). Transcriptomic analysis of *C9orf72* knockout mouse microglia revealed gene expression changes consistent with an inflammatory phenotype and correlated with increased microglia-mediated synaptic loss, which together imply that loss of *C9orf72* results in a proinflammatory and neurotoxic microglia phenotype (Lall et al., 2021). More recent studies using microglia generated from induced pluripotent stem cells (iPSCs) derived from ALS/FTD patients with *C9orf72* amplifications have shown that *C9orf2*-mutated human microglia exhibit typical hallmarks of *C9orf72* HRE pathology such as G_4C_2-repeat RNA foci, expression of dipeptide repeat proteins, and decreased expression level of C9orf72 (Lazzarini et al., 2023; Vahsen et al., 2023). Moreover, lipopolysaccharide (LPS)-primed *C9orf72*-mutated microglia were observed to have a pro-inflammatory phenotype and to be toxic to motor neurons when compared to healthy and/or isogenic microglia as controls (Banerjee et al., 2023; Vahsen et al., 2023).

Recent studies in both animal and human cell models have shown that the *C9orf72* HRE is associated with activation of the Nucleotide-Binding-Oligomerization Domain (NOD)-and Leucine-Rich Repeat (LRR)-containing (NLR) family, Pyrin-Domain-Containing 3 (NLRP3) inflammasome in microglia (Banerjee et al., 2023; Fu et al., 2023; Rivers-Auty et al., 2024; Shu et al., 2023; Trageser et al., 2023). The NLRP3 inflammasome is a cellular immune sensor that mediates pathological inflammation in autoimmune, metabolic, malignant, and neurodegenerative conditions. It detects stress signals, such as tissue damage or pathogen invasion, via immune cell components referred to as pattern recognition receptors (PRRs). For instance, the PRR Toll-like Receptor 4 (TLR4) can detect 'danger signals' and respond by enlisting Nuclear Factor-kappaB (NF-κB) to activate the transcription of genes encoding proteins involved in the NLRP3 inflammasome, including *NLRP3* itself. The NLRP3 protein assembles with other components, such as NIMA Related Kinase 7 (NEK7) and Apoptosis-associated Speck-like protein containing a Caspase recruitment domain (ASC), to form the active inflammasome complex. The NLRP3 complex activates Caspase-1 which in turn induces the release of inflammatory cytokines like Interleukin-1 beta (IL-1β) and IL-18 and the activation of pyroptosis, a highly inflammatory form of lytic cell death (Fu and Wu, 2023; Sharma and Kanneganti, 2021). Little is known, however, about the molecular mechanisms underlying NLRP3 inflammasome activation in *C9orf72*-mutated microglia.

In this study, we characterized the transcriptomic changes associated with *C9orf72* amplification in microglia derived from *C9orf2*-mutated iPSCs, and isogenic control iPSCs, using three different microglia differentiation methods. We specifically compared derivation protocols that generate microglia with distinct intrinsic inflammatory profiles to alleviate the possibility that differentiation protocol-specific basal activation of the induced microglia might confound the study of the impact of *C9orf72* mutation on inflammatory mechanisms. Here we show a consistent dysregulation of genes involved in pathways underlying NLRP3

inflammasome activation in *C9orf72*-mutated microglia generated using different methods. Consistently, conditioned media from *C9orf72*-mutated microglia enhance the death of *C9orf72*-mutated motor neurons *in vitro*. We hypothesize that cell-autonomous alterations in gene expression associated with activation of the NLRP3 inflammasome in *C9orf72*-mutated microglia contribute to the death of motor neurons in ALS. The transcriptomic data generated by this study would also serve as an objective source of hypothesis-generating data that may reveal novel mechanisms of microglial involvement in *C9orf72*-mutated ALS.

2. Results

2.1. Generation of C9orf72-mutated iPSC-derived microglia using three different derivation protocols

Activation of the NLRP3 inflammasome in microglia was observed in both mouse and human cells harbouring the *C9orf72* HRE (Banerjee et al., 2023; Fu et al., 2023; Rivers-Auty et al., 2024; Shu et al., 2023; Trageser et al., 2023). Little information is available, however, about the identity of genes that are dysregulated as part of the NLRP3 inflammasome activation in *C9orf72*-mutated microglia. To address this gap in knowledge, we implemented a comprehensive RNA sequencing (RNAseq) study of human microglia derived from *C9orf72*-mutated iPSC lines, and matching isogenic lines. Four previously studied (Chi et al., 2023; Ho et al., 2021; Gao et al., 2024; Thiry et al., 2024) iPSC lines were used for microglia derivation: the *C9orf72*-mutated iPSC lines CS52iALS-C9n6 (CS52-C9n6-M hereafter) and CS29iALS-C9n1 (CS29-C9n1-M), and their matching isogenic (ISO) control lines CS52iALS-C9n6.ISOxx (CS52-C9n6-ISO) and CS29iALS-C9n1.ISOxx (CS29-C9n1-ISO). Previous studies have demonstrated the presence of *C9orf72* HRE RNA foci and dipeptide repeat expression in neuromuscular organoids generated using these *C9orf72*-mutated iPSC lines (Gao et al., 2024), providing evidence that they can recapitulate *C9orf72* HRE-associated pathological features *in vitro*.

We generated iPSC-derived microglia using three separate differentiation methods. The use of multiple derivation protocols was aimed at mitigating the confounding effects that might result from the variability in the basal properties of iPSC-derived microglia generated using different *in vitro* differentiation protocols (Tang et al., 2022). Hereafter, we shall operationally refer to the three microglia differentiation methods used in the present study as protocols P1 (Douvaras et al., 2017), P2 (McQuade et al., 2018), and P3 (Haenseler et al., 2017) (Figure 1A–C). With

all protocols, we allowed microglia to acquire a mature phenotype *in vitro* by differentiating the cells for at least as long as indicated in the original publications and then confirming robustness and maturity of the induced cultures as we have previously described (Tang et al., 2022).

Examining isogenic iPSC lines CS52-C9n6-ISO and CS29-C9n1-ISO, we observed that all three microglia derivation protocols generated cultures highly enriched in cells expressing typical microglia/macrophage markers, including the protein IBA1, by immunocytochemistry (Figure 1D–F). More importantly, RNAseq data showed that the majority of 49 previously characterized microglia/macrophage cell marker genes (Butovsky et al., 2014; Hickman et al., 2013; Muffat et al., 2016; Zhang et al., 2014) were upregulated in microglia generated with all three protocols, when compared to other undifferentiated iPSC lines (Figure 1G–I).

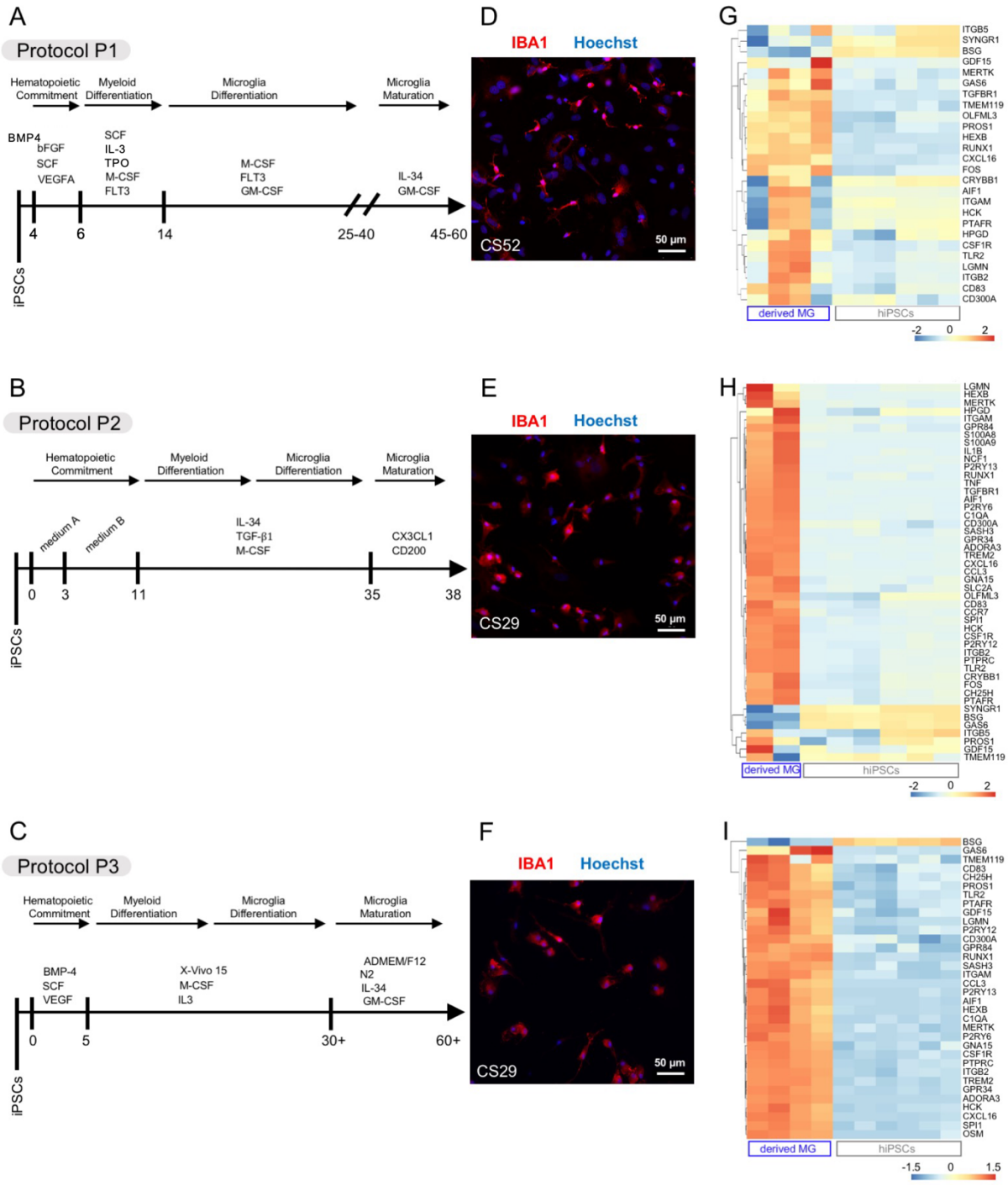


Figure 1. Generation and characterization of iPSC-derived microglia using three separate methods. (A–C) Schematics of three microglia differentiation protocols, referred to as "protocol P1" adapted from Douvaras et al. (A), "protocol P2" adapted from McQuade et al. (B), and "protocol P3" adapted from Haenseler et al. (C). (D–F) Representative images of microglia preparations differentiated from iPSC lines CS52-C9n6-ISO (CS52) and CS29-C9n1-ISO (CS29) with protocol P1 (D), P2 (E), or

P3 (F) and immunostained with anti-IBA1 antibody (red) and counterstained with Hoechst (blue). Scale bar, 50 μm. (G–I) Heatmaps showing known microglia/macrophage marker genes expression in undifferentiated iPSCs or microglia preparations differentiated from the CS52 and CS29 iPSC lines with protocol P1 (G), P2 (H) or P3 (I). Each column corresponds to one sample (undifferentiated iPSCs (hiPSCs) or isogenic iPSC-derived microglia (derived MG), as indicated at the bottom, and each line corresponds to one gene. Subgroups of genes with correlated expression patterns are portrayed by the dendrogram on the left side of each heatmap. Relative levels of gene expression are indicated by a color scale, with cool colors corresponding to low expression and warm colors corresponding to increased/high expression. Hierarchical clustering of rows was applied to the heatmaps to group genes with correlated expression patterns, resulting in different gene lists, depending on the samples analyzed in the given heatmap. The gene list used for this analysis was as follows: *HEXB, P2RY12, CXC3CR1, P2RY13, TREM2, S100A8, TMEM119, S100A9, RNASE4, GPR34, FCRLS, SIGLECH, OLFML3, FOS, SCLO2B1, TGFBR1, SLC2A5, CAMP, ITGB5, CRYBB1, SYNGR1, GPR56, NGP, CMRF35, HPGD, P2RY12, GPR34, MERTK, C1QA, PROS1, GAS6, ITGAM, ITGB2, CSF1R, PTPRC, AIF1, ADORA3, LGMN, GPR84, CCR7, BCL2A1D, TNF, NCF1, GDF15, OSM, LMC25, H2-OA, CD83, CCL3, GNA15, IL1B, PFAU, CCL9, TMEM119, C1QA, LRF8, CXCL16, CH25H, HCK, CCL12, PTAFR, CD300A, LRT5, STPI1, SELPIG, SASH3, BSG, TLR2, P2RY6, CDI4, BCL2A1A, BCL2A1C, RUNX1, SPI1*. Abbreviations: hiPSC, human iPSC; MG, microglia.

Microglia generated using protocol P2 had the highest number of significantly upregulated genes encoding pro-inflammatory molecules, such as Chemokine (C-C motif) ligand 3 (CCL3), C-C Motif Chemokine Receptor 7 (CCR7), C-X-C Motif Chemokine Ligand 16 (CXCL16), G Protein Coupled Receptor 84 (GPR84), Hematopoietic Cell Kinase (HCK), Interleukin-1beta (IL-1β), S100 Calcium Binding Protein A9 (S100A9), S100 Calcium Binding Protein B (S100B), Tumor Necrosis Factor (TNF), when compared to microglia generated with protocols P1 and P3 (Figure 1I-G). Consistently, direct comparison of the expression of 49

microglia/macrophage genes (Butovsky *et al.,* 2014; Hickman *et al*., 2013; Muffat *et al.*, 2016; Zhang *et al*., 2014) across a panel of RNAseq datasets from microglia generated using the different three methods showed that protocol 2-microglia had the highest levels of pro-inflammatory genes. Protocol P3 had the lowest levels of these genes, suggesting that method 3-microglia are the least developmentally mature and/or least activated compared to microglia obtained using the other two protocols, even after longer *in vitro* differentiation times (Figure 2). Together, these observations suggest that microglia generated using method P2 are more pro-inflammatory than microglia obtained using protocols P1 and P3 under comparable experimental conditions.

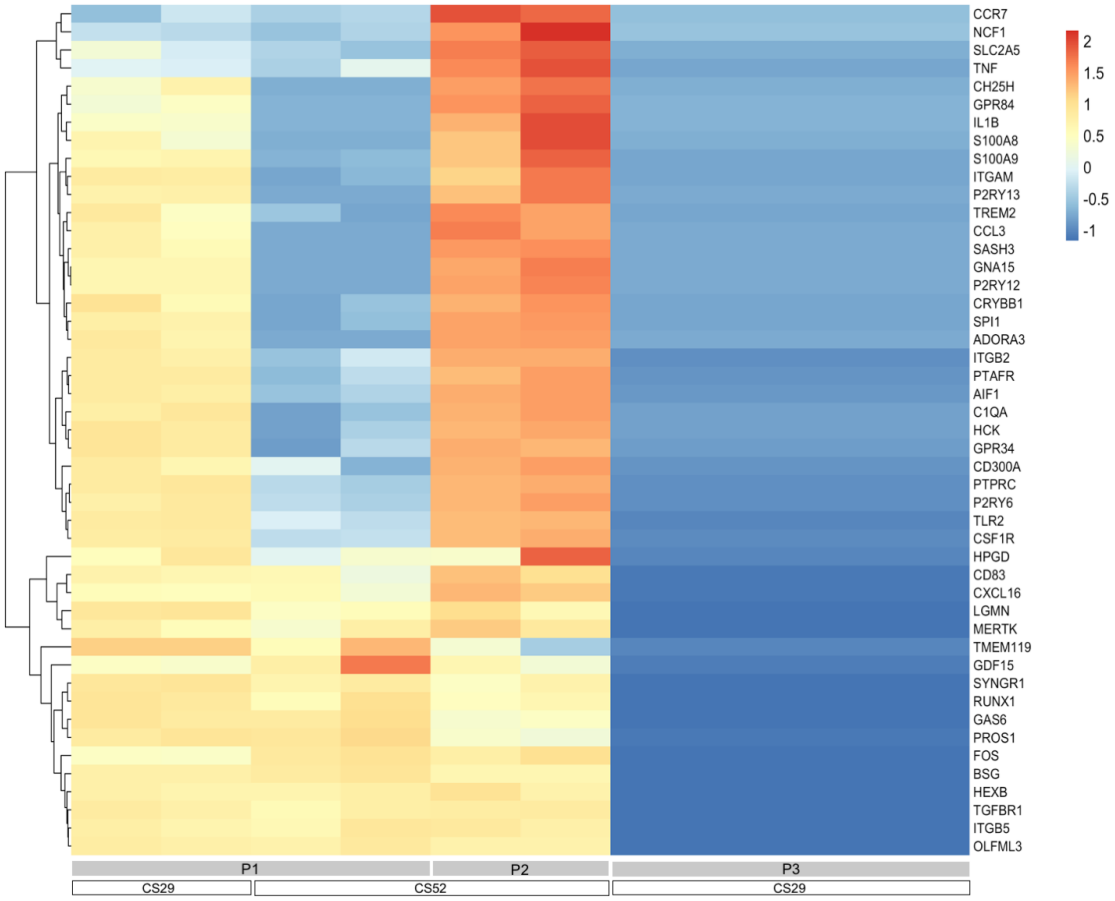


Figure 2. Comparison of microglial/macrophage genes expression in iPSC-derived microglia obtained with three different protocols. Heatmap showing known microglia/macrophage marker

genes expression in microglia preparations differentiated from the CS52-C9n6-ISO (CS52) and CS29-C9n1-ISO (CS29) iPSC lines with protocols P1, P2, or P3. Each column corresponds to one isogenic sample (generated from the CS52 or the CS29 iPSC line with protocols P1, P2 or P3, as indicated at the bottom) and each line corresponds to one gene. Subgroups of genes with correlated expression patterns are portrayed by the dendrogram on the left side of the heatmap. Relative levels of gene expression are indicated by a color scale, with cool colors corresponding to low expression and warm colors corresponding to increased/high expression. The gene list used for this analysis was the same as the list shown in the legend to Figure 1.

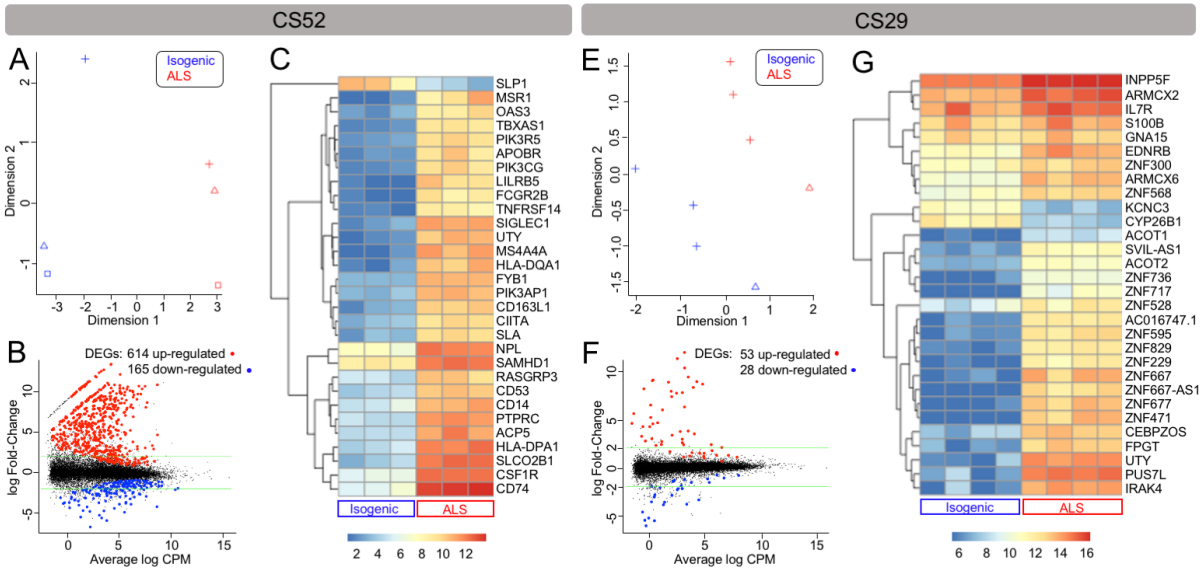
2.2. Dysregulated transcriptome of C9orf72-mutated iPSC-derived microglia

All iPSC lines, *C9orf72*-mutated and isogenic, were used to generate microglia using at least two of the three derivation methods described above. All microglia preparations (25 in total) were analysed by RNAseq. To search for intrinsic transcriptomic changes associated with the *C9orf72* HRE, all microglia preparations were not exposed to lipopolysaccharide (LPS) or other treatments inducing microglia activation prior to RNA collection.

We first analyzed RNA isolated from three separate microglia cultures derived from the *C9orf72*-mutated iPSC line CS52-C9n6-M and three microglia cultures from the matching isogenic control line CS52-C9n6-ISO. These 6 biological samples were obtained using differentiation protocol P1, which in our hands generates microglia that are less intrinsically pro-inflammatory than microglia generated using method 2 (Figure 2). Principle component analysis showed that CS52-C9n6-M microglia grouped together along one dimension, clustering away from CS52-C9n6-ISO microglia (Figure 3A). Comparing the transcriptomic data from these six separate preparations using an EdgeR (version 4.0) analysis pipeline for differentially expressed gene (DEG) analysis (Chen *et al.*, 2016), we identified 614 genes that were significantly upregulated in *C9orf72*-mutated microglia, while 165 genes were

downregulated (adjusted p-value (false discovery rate; FDR) <0.05, with no fold-change cutoff) (Figure 3B). The top 30 most upregulated DEGs in CS52-C9n6-M microglia included several genes that have previously been associated with ALS, such as *Cluster of Differentiation 14* (*CD14*), *Class II Major Histocompatibility Complex Transactivator* (*CIITA*), *Fc Gamma Receptor IIb* (*FCGR2B*), *Major Histocompatibility Complex, Class II, DQ Alpha 1* (*HLA-DQA1*), and *Colony Stimulating Factor 1 Receptor* (*CSF1R*) (Beers et al., 2022; Chi et al., 2023; Martinez-Muriana et al., 2016; Sanfilippo et al., 2017; Xie et al., 2021) (Figure 3C). Several genes involved in microglia/macrophage activation were also present within the top 30 DEGs, including *Cluster of Differentiation 74* (*CD74*), *Macrophage Scavenger Receptor 1* (*MSR1*), *Phosphoinositide-3-Kinase Regulatory Subunit 5* (*PIK3R5*), *Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Gamma* (*PIK3CG*), to name a few (Gu et al., 2024; Ji et al., 2022; Lajqi et al., 2019; Peferoen et al., 2015).

Importantly, a number of the top 30 DEGs (*CD14*, *CIITA*, *CSF1R*, *PIK3CG*, *Protein Tyrosine Phosphatase Receptor Type C* (*PTPRC*)*, Sialic Acid Binding Ig Like Lectin 1* (*SIGLEC1*; also known as *Cluster of Differentiation 169* (CD169)) encoded proteins previously implicated in the modulation of pyroptosis mechanisms, including the NLRP3 inflammasome (Figure 3C) (Du et al., 2020; Lu et al., 2023; Radian et al., 2015; Souza de Lima et al., 2017; Vasudevan et al., 2022; Vidmar et al., 2019). In agreement with these observations, combined Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of the upregulated DEGs in CS52-C9n6-M microglia generated using protocol P1 revealed enrichment in processes associated with immune response, phagocytosis, and innate immunity, to name a few (Figure 3D). The latter finding resembles the observation by Vahsen and colleagues that LPS-primed *C9orf72*-mutated iPSC-derived microglia (obtained using a protocol resembling protocol P3 in our study) exhibit enrichment in DEGs involved in pathways associated with immune-cell activation (Vahsen et al., 2023).



D

Functional Cat.	Terms	FDR	Fold. Enrich.
Over-represented in ALS			
KEGG Pathways	Phagosome	2.074e-17	6.43
	Osteoclast differentiation	7.748e-13	5.764
	Intestinal immune network for IgA production	1.168e-12	10.579
	Cell adhesion molecules	8.929e-12	5.213
	Hematopoietic cell lineage	1.622e-11	6.614
	Neutrophil extracellular trap formation	7.794e-8	3.837
	Antigen processing and presentation	2.642e-7	5.798
	Toll-like receptor signaling pathway	7.626e-7	4.756
Biological Process	immune response	6.197e-30	5.39
	inflammatory response	6.33e-26	5.516
	innate immune response	4.103e-18	4.143
	antigen processing/presentation of exogenous antigen via MHC class II	7.482e-18	21.951
	positive regulation of tumor necrosis factor production	1.259e-17	9.593
	positive regulation of interleukin-6 production	3.249e-12	8.345
	cell surface receptor signaling pathway	1.017e-10	4.24
	peptide antigen assembly with MHC class II protein complex	5.94e-10	24.623
	adaptive immune response	2.668e-9	3.407
	positive regulation of T cell activation	4.02e-9	13.195
	integrin-mediated signaling pathway	5.833e-9	6.837
	signal transduction	5.926e-9	2.253
	positive regulation of ERK1 and ERK2 cascade	9.513e-9	4.555
	positive regulation of T cell proliferation	1.501e-8	9.394
	neutrophil chemotaxis	1.774e-8	7.767
	positive regulation of interleukin-12 production	1.947e-8	11.395
	antigen processing and presentation	2.521e-8	11.142
	positive regulation of phagocytosis	3.07e-8	9.768
	cellular response to lipopolysaccharide	1.01e-7	4.688
	B cell receptor signaling pathway	1.232e-7	8.807
	toll-like receptor signaling pathway	2.617e-7	14.07
	defense response to bacterium	5.15e-7	4.894
	chemotaxis	7.684e7	5.624
	phagocytosis	8.565e-7	7.566
	regulation of cell shape	9.575e-7	5.19
	positive regulation of inflammatory response	9.575e-7	5.861
Cellular Component	plasma membrane	8.339e-34	1.92
	external side of plasma membrane	7.37e-23	5.161
	cell surface	9.25e-16	3.539
	secretory granule membrane	3.012e-12	8.321
	membrane	3.012e-12	1.586
	MHC class II protein complex	1.956e-9	17.032
	tertiary granule membrane	5.137e-9	8.594
	lysosome	5.752e-9	3.916
	specific granule membrane	1.259e-7	6.894
Molecular Function	transmembrane signaling receptor activity	3.746e-11	5.764
	MHC class II protein complex binding	1.143e-9	16.838
	MHC class II receptor activity	1.185e-7	24.211
	peptide antigen binding	4.698e-6	7.65
	signaling receptor activity	4.772e-6	3.886
	virus receptor activity	6.735e-6	6.557
Under-represented in ALS			
KEGG Pathways	p53 signaling pathway	1.119e-8	17.249
Biological Process	negative regulation of cell population proliferation	5.201e-2	4.222
	intrinsic apoptotic signaling pathway in response to DNA damage by p53 class mediator	5.201e-2	21.357
	cell migration	7.284e-2	5.016
	negative regulation of apoptotic process	7.98e-2	3.614
Cellular Component	plasma membrane	8.197e-4	1.674
	tight junction	4.505e-3	25.723
	bicellular tight junction	2.593e-3	7.525

H

Functional Cat.	Terms	FDR	Fold. Enrich.
Over-represented in ALS			
Biological Process	regulation of DNA-templated transcription	1.07e-4	6.279
	regulation of transcription by RNA polymerase II	4.37e-3	3.761
	cellular response to lipopolysaccharide	0.743	9.339
Cellular Component	nucleus	0.895	1.61
Molecular Function	RNA polymerase II cis-regulatory region sequence-specific DNA binding	1.48e-5	5.593
	metal ion binding	1.09e-2	2.664
	DNA-binding transcription factor activity, RNA polymerase II-specific	6.07e-2	3.476
	thiolester hydrolase activity	0.318	178.68
	RAGE receptor binding	0.557	81.218
	fatty acyl-CoA hydrolase activity	0.589	63.814
	JUN kinase kinase kinase activity	0.657	44.67
	MAP kinase kinase kinase activity	0.657	42.543
	carboxylic ester hydrolase activity	0.959	25.526
Under-represented in ALS			
Biological Process	cell-cell adhesion	0.413	16.02
Cellular Component	plasma membrane	1.17e-3	2.645
	glutamatergic synapse	0.922	5.848
	cell-surface	0.922	5.179

I

DEGs common to CS52 and CS29

Genes Up-regulated in both lines	pValue in CS52	pValue in CS29	log Fold Change in CS52	log Fold Change in CS29
UTY	3.912002e-06	1.322923e-08	10.7781841	8.8531482
CD14	4.646790e-06	9.685961e-05	6.0116649	1.2148671
ZNF736	2.015265e-06	5.254785e-06	9.3487203	6.4277039
S100B	2.713710e-05	5.381982e-06	10.1753122	1.4350439
TRIM4	8.153832e-05	1.738991e-04	9.6295279	1.1248935
INPP5F	1.038843e-04	1.058438e-08	1.7689973	2.0276213
SLAMF8	2.942391e-04	8.138939e-05	6.7753227	1.4328724
VSIG4	3.815600e-04	1.171606e-04	8.9936900	1.2317889
S100A9	5.105895e-04	5.711180e-05	9.3158154	1.4928877
ZNF528	5.744346e-04	2.707144e-06	2.5074243	3.3020958
GNAS-AS1	5.744346e-04	6.760163e-05	4.7964892	4.1957278
GNA15	7.362756e-04	1.703414e-05	11.2058223	1.1502714
ZNF717	8.813501e-04	8.257522e-06	6.7292153	8.9533591
HLA-DQA2	1.027702e-03	7.865046e-05	10.5620790	2.9321788
FPGT	1.226576e-03	1.246918e-07	10.4622403	7.7176783
ZNF829	1.590837e-03	8.233795e-09	2.3598436	7.8573645
ASNSP1	1.693452e-03	5.896647e-05	5.8938203	9.0220488
MNS1	1.960391e-03	8.904631e-05	9.5565706	2.2415935

Genes Down-regulated in CS29	pValue in CS52	pValue in CS29	log Fold Change in CS52	log Fold Change in CS29
MYH14	3.316398e-04	8.521301e-05	4.1010659	-6.0890449
ITGB6	9.075363e-04	1.802274e-04	5.1897171	-3.4076359

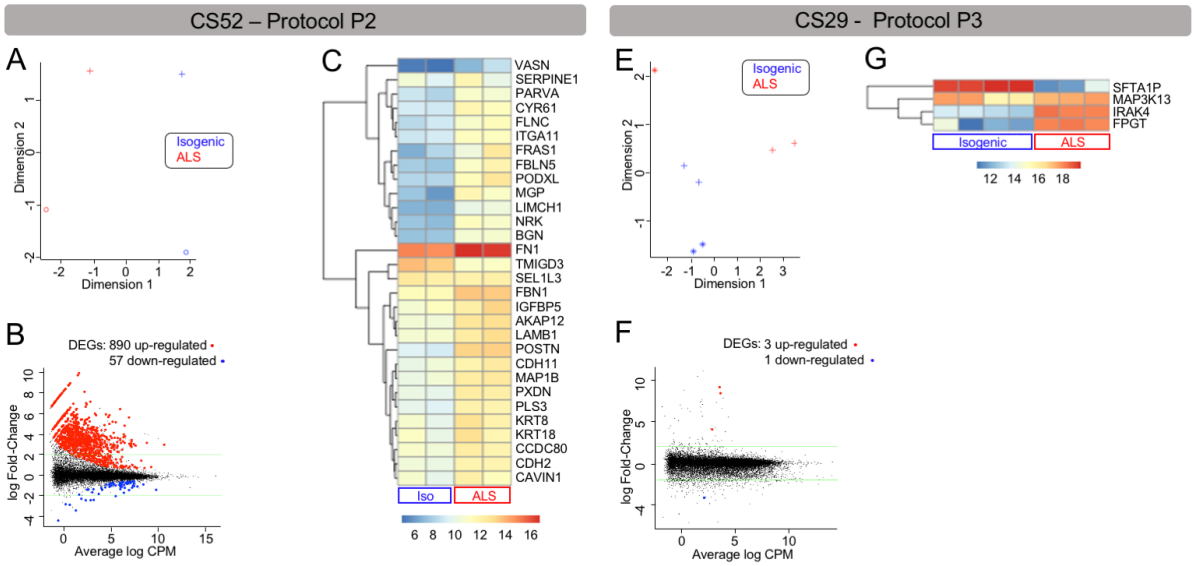
Figure 3. Differential gene expression between corrected isogenic and *C9orf72*-mutated microglia derived using protocol P1. (A–H**)** Differential gene expression and gene ontology analysis of CS52-C9n6-M versus CS52-C9n6-ISO microglia (CS52 collectively) (A–D) or CS29-C9n1-M versus CS29-C9n1-ISO microglia (CS29 collectively) (E–H). (A and E) Principal component analysis plots showing the relationship between the transcriptomic profiles of CS52 (A) or CS29 (E) iPSC-derived microglia. Each plotted data point represents one biological replicate, and the different symbols refer to the different batches. Blue data points correspond to corrected isogenic samples (Isogenic); red data points correspond to *C9orf72*-mutated samples (ALS). (B and F) Mean-difference plots (MA plots) showing the average log of Count per Million (CPM; X-axis) and log Fold Changes (Y-axis), highlighting the differentially expressed genes (DEGs) in *C9orf72*-mutated (ALS) compared to corrected isogenic microglia differentiated from the CS52 (B) or CS29 (F) iPSC lines. Each plotted data point represents one gene: black data points correspond to genes that are not DEGs, red data points correspond to up-regulated DEGs, and blue data points correspond to down-regulated DEGs (p-adjusted <0.05, with no Fold Change cutoff). The green lines indicate 2-fold up or down. The numbers of up- and down-regulated DEGs are indicated on top of each MA plot. (C and G) Heatmaps of the topmost DEGs in *C9orf72*-mutated (ALS) compared to corrected isogenic microglia differentiated from the CS52 (C) or CS29 (G) iPSC lines. For each heatmap, each column corresponds to one sample (Isogenic or ALS, as indicated at the bottom) and each line corresponds to one gene. Relative levels of gene expression are indicated by a color scale, with cool colors corresponding to low expression and warm colors corresponding to increased/high expression. (D and H) Gene ontology analysis tables showing KEGG pathways, biological processes, cellular components and molecular functions that are significantly over- or under-represented in *C9orf72*-mutated (ALS) compared to isogenic microglia differentiated from either the CS52 (D) or CS29 (H) iPSC line. (I) Table showing the 20 DEGs common to CS52-C9n6-M and CS29-C9n1-M microglia. The p value and log Fold Change are indicated for each gene.

When the same analysis was conducted using eight RNAseq datasets from microglia derived from *C9orf72*-mutated iPSC line CS29-C9n1-M and its matching isogenic control CS29-C9n1-ISO

differentiated using protocol P1, we identified 53 significantly upregulated genes in *C9orf72*-mutated microglia, while 28 genes were downregulated (Figure 3E, F). Interestingly, isogenic microglia generated from CS29-C9n1-ISO iPSCs appeared intrinsically more inflammatory than microglia generated from CS52-C9n6-ISO (Figure 2). The list of top 30 DEGs in CS29-C9n1-M microglia revealed predominantly gene-regulation- and proliferation-associated genes, including numerous zinc finger protein-encoding genes (Figure 3G). The observation of several genes encoding zinc finger-domain proteins among the top DEGs agrees with the identification of more than 20 zinc-finger family members as genes implicated in inflammation in ALS from blood transcriptome profiles (Pappalardo et al., 2024). GO and KEGG analysis of the upregulated DEGs in CS29-C9n1-M microglia revealed enrichment in processes associated with transcription, DNA binding, RNA polymerase II activity, but no significant enrichment in immune processes apart from the term 'response to LPS' (Figure 3H). However, when we searched for DEGs common to both CS52-C9n6-M and CS29-C9n1-M microglia derived using protocol P1, we identified 18 significantly upregulated DEGs in both cases, as well as two common DEGs that were upregulated in CS52-C9n6-M microglia but downregulated in CS29-C9n1-M microglia (Figure 3I). These 20 common DEGs include 6 genes implicated in NLRP3 inflammasome-mediated mechanisms: *CD14*, *Integrin Subunit Beta 6* (*ITGB6*), *S100A9*, *S100B*, *SLAM Family Member 8* (*SLAMF8*), and *V-Set and Immunoglobulin Domain Containing 4* (*VSIG4*) (Arterbery et al., 2018; Bi et al., 2019; Huang et al., 2019; Li et al., 2025; Pruenster et al., 2023; Vizuete et al., 2022; Wang et al., 2024). This finding further suggests that mechanisms involving the NLRP3 inflammasome are dysregulated in *C9orf72*-mutated microglia.

The commonality of 20 DEGs in microglia that exhibit varying degrees of mutation-associated alterations in gene expression suggests that at least some of these transcriptomic changes may represent biologically relevant DEGs in *C9orf72*-mutated microglia. To test this possibility, and in the process search for additional DEGs, we next investigated microglia generated from

CS52-C9n6-M iPSCs, and matching isogenic iPSCs, using differentiation protocol P2. As mentioned, this derivation method gives rise to microglia that appear more activated and inflammatory than those obtained with protocols 1 and 3 in our hands (Figure 2). We identified 890 genes significantly upregulated in CS52-C9n6-M microglia, with 57 genes significantly downregulated (Figure 4A, B). Perhaps not surprisingly given the phenotypic differences between microglia generated using protocols P1 and P2, the top 30 DEGs in CS52-C9n6-M microglia generated with differentiation method P2 were mainly different from the top 30 DEGs identified using differentiation method P1(Figure 4C). Analysis of molecular and cellular pathways associated with up-regulated DEGs revealed mechanisms converging on cell adhesion, extracellular matrix organization, and cell-matrix adhesion (Figure 4D). Nevertheless, genes associated with NLRP3 inflammasome components were also present among the top 30 DEGs identified in CS52-C9n6-M microglia generated with protocol P2: these included *Biglycan* (*BGN*), *Caveolae Associated Protein 1* (*CAVIN1*) also termed *Polymerase 1 and Transcript Release Factor* (*PTRF*), *Cystein Rich Angiogenic Inducer 61* (*CYR61*) also known as *Cellular Communication Network Factor 1* (*CCN1*), and *Periostin* (*POSTN)* (Babelova et al., 2009; Bai et al., 2010; Jin et al., 2024; Yao et al., 2020; Zheng et al., 2013; Zhou et al., 2021) (Figure 4C). Although not among the top 30 DEGs, *SIGLEC1* and *ITGB6* were also among the among the significantly upregulated genes in CS52-C9n6-M microglia generated with protocol P2, in agreement with protocol P1 RNAseq data. These findings further suggest that genes involved in NLRP3 inflammasome/pyroptosis biology are significant DEGs in *C9orf72*-mutated ALS microglia.



D

Functional Cat.	Terms	FDR	Fold. Enrich.
Over-represented in ALS			
KEGG Pathways	Cytoskeleton in muscle cells	1.898e-27	5.767
	Focal adhesion	1.589e-17	4.971
	ECM-receptor interaction	5.079e-17	7.640
	PI3K-Akt signaling pathway	1.628e-10	3.030
	Pathways in cancer	3.017e-8	2.387
	Hippo signaling pathway	5.041e-8	3.912
Biological Process	cell adhesion	1.201e-21	3.855
	cell-cell adhesion	1.997e-20	5.794
	extracellular matrix organization	5.345e-15	5.617
	axon guidance	7.257e-14	4.911
	cell migration	1.142e-12	4.079
	angiogenesis	2.0e-11	4.072
	collagen fibril organization	2.334e-10	8.05
	homophilic cell adhesion via plasma membrane adhesion molecules	2.730e-10	4.530
	nervous system development	3.762e-10	3.130
	multicellular organism development	1.737e-9	6.767
	cell-matrix adhesion	1.837e-8	5.436
	positive regulation of phosphatidylinositol 3-kinase/protein kinase B signal transduction	4.756e-8	4.023
Cellular Component	collagen-containing extracellular matrix	5.993e-33	5.150
	focal adhesion	5.271e-25	4.370
	basement membrane	1.555e-23	9.541
	plasma membrane	2.034e-23	1.610
	extracellular region	1.177e-21	2.143
	extracellular matrix	1.960e-20	5.315
	adherens junction	1.559e-16	5.279
	endoplasmic reticulum lumen	3.045e-16	4.182
	cell surface	1.159e-12	2.741
	cell-cell junction	2.276e-12	4.666
	stress fiber	2.276e-12	7.026
	Z disc	4.531e-11	5.219
	extracellular space	3.211e-10	1.772
	extracellular exosome	3.794e-8	1.639
	sarcolemma	3.967e-8	5.143
	dendrite	1.140e-7	2.610
	glutamatergic synapse	1.285e-7	2.663
	actin cytoskeleton	2.388e-7	3.116
	cytoskeleton	5.511e-7	2.414
	axon	5.899e-7	2.699
	receptor complex	6.199e-7	3.335
Molecular Function	extracellular matrix structural constituent	1.901e-25	8.780
	calcium ion binding	1.240e-19	3.042
	actin binding	8.573e-11	3.663
	extracellular matrix binding	3.503e-9	10.859
	integrin binding	4.815e-9	4.424
	actin filament binding	1.057e-8	3.629
Under-represented in ALS			
KEGG Pathways	p53 signaling pathway	2.145e-4	27.2
Cellular Component	plasma membrane	2.388e-3	2.158
	cell surface	1.992e-2	5.652
	external side of plasma membrane	6.487e-2	6.492

H

Functional Cat.	Terms	FDR	Fold. Enrich.
Over-represented in ALS			
Biological Process	JNK cascade	0.197	215.672
Cellular Component	cytoplasm	0.683	3.630
Molecular Function	protein kinase activity	0.295	46.460
	protein serine kinase activity	0.295	35.162
	protein serine/threonine kinase activity	0.295	33.689
	protein kinase binding	0.295	25.636

Figure 4. Differential gene expression between corrected isogenic and *C9orf72*-mutated microglia derived using protocols P1 and P2. (A–H) Differential gene expression and gene ontology analysis of CS52-C9n6-M versus CS52-C9n6-ISO microglia (CS52 collectively) generated using protocol P2 (A–D) or CS29-C9n1-M versus CS29-C9n1-ISO microglia (CS29 collectively) generated using protocol P3

(E–H). (A and E) Principal component analysis plots showing the relationship between the transcriptomic profiles of CS52 microglia generated with protocol P2 (A) or CS29 microglia generated with protocol P3 (E). Each plotted data point represents one biological replicate, and the different symbols refer to the different batches. Blue data points correspond to isogenic samples (Isogenic); red data points correspond to *C9orf72*-mutated samples (ALS). (B and F) Mean-difference plots (MA plots) showing the average log of Count per Million (CPM; X-axis) and log Fold Changes (Y-axis), highlighting the differentially expressed genes (DEGs) in *C9orf72*-mutated (ALS) compared to corrected isogenic microglia differentiated from the CS52 (B) or CS29 (F) iPSC lines using protocols P2 or P3, respectively. Each plotted data point represents one gene: black data points correspond to genes that are not DEG, red data points correspond to up-regulated DEGs, and blue data points correspond to down-regulated DEGs (p-adjusted <0.05, logFC >0.26). The green lines indicate 2-fold up or down. The numbers of up- and down-regulated DEGs are indicated on top of each MA plot. (C and G) Heatmaps of the topmost DEGs in *C9orf72*-mutated (ALS) compared to isogenic microglia differentiated from either the CS52 iPSC line with protocol P2 (C) or the CS29 iPSC line with protocol P3 (G). For each heatmap, each column corresponds to one sample (Isogenic or ALS, as indicated at the bottom) and each line corresponds to one gene. Relative levels of gene expression are indicated by a color scale, with cool colors corresponding to low expression and warm colors corresponding to increased/high expression. (D and H) Gene ontology analysis tables showing KEGG pathways, biological processes, cellular components and molecular functions that are significantly over- or under-represented in *C9orf72*-mutated compared to isogenic microglia differentiated from either the CS52 iPSC line with protocol P2 (D) or CS29 iPSC line with protocol P3 (H).

To examine the latter possibility further, we next generated microglia from CS29-C9n1-M and CS29-C9n1-ISO iPSCs using protocol P3. As mentioned above, in our hands this method generates microglia that appear less developmentally mature and less inflammatory than those obtained using the other two methods. Although only 4 DEGs were identified in CS29-C9n1-

M microglia generated using protocol P3 (Figure 4E–H), one of these DEGs, *IL-1 Receptor–Associated Kinase 4* (*IRAK4*), encodes a protein previously shown to be involved in the NLRP3 inflammasome (Leaf et al., 2017). Of note, *IRAK4* was also identified as a top 30 DEG in CS29-C9n1-M microglia generated using protocol P1 (Figure 3G). Interestingly, a second DEG was also of interest in the context of glial cell activation and caspase-mediated mechanisms of cell death, namely *Mitogen-Activated Protein Kinase Kinase Kinase 13* (*MAP3K13*, also known as *Leucine Zipper-Bearing Kinase* (*LZK*). MAP3K13/LZK promotes astrocyte reactivation and can activate NF-κB signaling (Chen et al., 2018; Masaki et al., 2003). One additional DEG was conserved in CS29-C9n1-M microglia generated using protocols P1 and P3, namely *Fucose-1-Phosphate Guanylyltransferase* (*FPGT*) (Figures 3G and 4G). *FPGT* has previously been implicated in inflammation in ALS from blood transcriptome profiles (Pappalardo et al., 2024).

In addition to the above-discussed genes, three other genes were consistently identified as top DEGs associated with the *C9orf72* mutation in all 25 RNAseq datasets, namely *Protocadherin Alpha 2* (*PCDHA2*) (FDR, 3.135e-2; fold enrichment, 4.381), *Asparagine Synthetase Pseudogene 1* (*ASNSP1*) (FDR, 4.2e-3; fold enrichment, 6.495), and *AC004846.1* (FDR, 4.2e-3; fold enrichment, 4.049). Of note, *AC004846.1* encodes a long non-coding RNA (lncRNA) identified as a pyroptosis-related lncRNA in colorectal cancer (Cai et al., 2022), further implicating alterations of inflammasome/pyroptosis mechanisms in *C9orf72*-mutated microglia. It is also worth mentioning that *PCDHA2* is a member of the *Protocadherin Alpha* gene cluster composed of 15 cadherin superfamily genes, including *PCDHA9*, which has recently been identified as a candidate gene for ALS (Zhong et al., 2024).

In summary, the combined analysis of all 25 RNAseq datasets from *C9orf72*-mutated and isogenic microglia, differentiated from different iPSC lines using different methods, identified several upregulated genes implicated in the NLRP3 inflammasome. These include genes encoding cell membrane proteins (CD14, CSFR1, PTPRC, SLAMF8), extracellular matrix

(ECM) components (BGN, CYR61, ITGB6, POSTN, SIGLEC1, VSIG4), intracellular kinases (IRAK4, PIK3CG), nuclear transcription factors (CIITA), intracellular proteins with secretory potential (S100A9, S100B), and proteins with broad intracellular distribution (CAVIN1). Taken together, the present results have characterized cell-autonomous changes in the transcriptome of *C9orf72*-mutated iPSC-derived microglia, consistent across different experimental conditions, providing evidence for dysregulation of mechanisms related to the NLRP3 inflammasome and implicating specific genes in these events.

2.3. Effect of C9orf72-mutated iPSC-derived microglia conditioned medium on motor neurons

The cell-autonomous transcriptomic changes observed in *C9orf72*-mutated microglia suggest that these cells can be toxic to motor neurons due to dysregulated NLRP3 inflammasome-activated mechanisms, which would lead to elevated release of neurotoxic factors. To determine whether *C9orf72*-mutated microglia secrete factors that are sufficient to decrease motor neuron survival, we examined the effect of conditioned media from either *C9orf72*-mutated microglia or their matching isogenic controls on both *C9orf72*-mutated and isogenic motor neurons. Mutated or isogenic motor neurons were differentiated for 28 days, followed by addition of either no conditioned media or conditioned media collected from *C9orf72*-mutated or isogenic microglia generated using protocol P1. Motor neurons were then cultured till day 49 and then subjected to immunocytochemistry with an antibody against cleaved caspase 3 (CC3) to detect apoptotic cells (Figure 5A). Motor neurons were visualized with an antibody against choline acetyltransferase (CHAT). With no conditioned media added, quantification of the numbers of CHAT-expressing cells positive for CC3 showed increased numbers of apoptotic *C9orf72*-mutated motor neurons compared to isogenic motor neurons (Figure 5B, bars 1 and 2) indicating intrinsic reduced viability of the mutant cells, in agreement with previous studies

(Thiry et al., 2024). Exposure to conditioned media from isogenic microglia had no detectable effect on the viability of either *C9orf72*-mutated or isogenic motor neurons (Figure 5B, bars 1–4). In contrast, we observed a statistically significant increase in the fraction of CC3-positive *C9orf72*-mutated motor neurons in the presence of conditioned media from *C9orf72*-mutated microglia compared to absence of any conditioned media or exposure to media from isogenic microglia (Figure 5B, cf. bars 2, 4, 6). Isogenic motor neuron cultures tended to contain more CC3-positive cells in the presence of conditioned media from *C9orf72*-mutated microglia, but this was not statistically significant when compared to conditioned media from isogenic microglia (Figure 5B, cf. bars 3 and 5). These results provide evidence that *C9orf72*-mutated microglia enhance the death of *C9orf72*-mutated motor neurons, and that this effect does not require motor neuron-microglial cell contact.

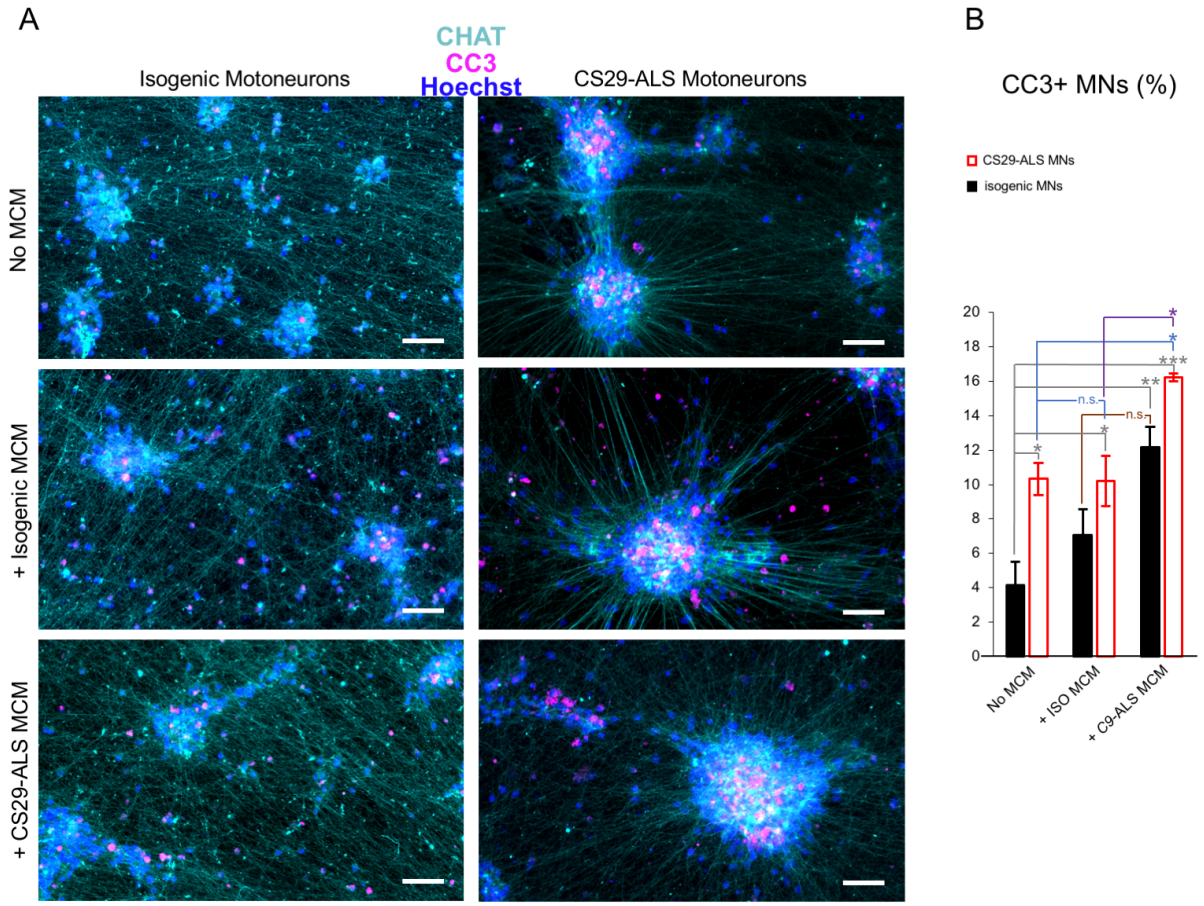


Figure 5. Effect of *C9orf72*-mutated microglia conditioned media on *C9orf72*-mutated motor neurons. (A) Representative immunocytochemistry images of motor neurons (Motoneurons) derived from isogenic (left column) or *C9orf72*-mutated (CS29-ALS) (right column) iPSCs and maintained in culture for 3 weeks in the absence of microglia conditioned media (MCM) (top images: No MCM), or in the presence of conditioned media from isogenic microglia (middle images: isogenic MCM) or *C9orf72*-mutated (CS29-ALS) MCM (bottom images). Cultures were immunostained with anti-CC3 antibody (magenta), anti-CHAT antibody (cyan), and counterstained with Hoechst (blue). Scale bars, 50 µm. (B) Bar plot showing the quantification of the percentage of apoptotic CC3-positive motor neurons in isogenic or *C9orf72*-mutated CS29 motor neuron preparations after 3 weeks of culture in each of the following three conditions: absence of MCM (No MCM); presence of isogenic microglia conditioned media (ISO MCM); or presence of *C9orf72*-mutated CS29 MCM (C9-ALS MCM). Statistics: two-way ANOVA and Tukey's multiple comparisons post-test; n.s. = non-significant; * = p value < 0.05; ** = p value < 0.005; *** = p value < 0.0005.

3. Discussion

There is consistent evidence from studies in animal and human cell models of ALS that the *C9orf72* HRE is associated with activation of the NLRP3 inflammasome in microglia (Banerjee et al., 2023; Fu et al., 2023; Rivers-Auty et al., 2024; Trageser et al., 2023). Little information is available, however, about the mechanisms underlying the activation of inflammasome-mediated processes in *C9orf72*-mutated microglia. The NLRP3 inflammasome detects stress signals, such as tissue damage or pathogen invasion, through immune cell components referred to as pattern recognition receptors. For instance, Toll-like Receptor 4 (TLR4) can detect 'danger signals' and respond through activation of NF-κB to activate the transcription of genes encoding proteins involved in the NLRP3 inflammasome, including *NLRP3* itself. The NLRP3 protein mediates inflammasome-mediated mechanisms by assembling with other components, such as NIMA Related Kinase 7 (NEK7) and apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), to form the active inflammasome complex. Active NLRP3 activates caspase-1 which in turn induces the release of inflammatory cytokines like IL-1β and IL-18 and activation of pyroptosis, a highly inflammatory form of lytic cell death (Fu and Wu, 2023; Sharma and Kanneganti, 2021).

The present study has identified several genes upregulated in *C9orf72*-mutated microglia. Elevated expression of a number of proteins encoded by these upregulated genes could cause dysregulation of TLR4/NF-κB-mediated activation of the NLRP3 inflammasome. For instance, DEG *SLAMF8* codes for a cell surface receptor that is highly induced in collagen-induced rheumatoid arthritis and knockout of *SLAMF8* attenuates this condition through inhibition of TLR4/NF-κB signaling (Qin et al., 2022). Consistently, simultaneous knockout of *SLAMF8* and its related family member *SLAMF9* prevents LPS-induced liver inflammation by downregulating TLR4 expression on macrophages (Zeng et al., 2020). These findings suggest

that abnormally elevated levels of SLAMF8 in *C9orf72*-mutated microglia might play a role in initiating inflammation and/or maintaining a pro-inflammatory environment via TLR4/NF-κB-mediated activation of the NLRP3 inflammasome. Another candidate activator of the NLRP3 inflammasome is the product of DEG *CD14*, which is a GPI-anchored co-receptor for TLRs involved LPS-induced responses. CD14/TLR coreceptors are important for microglia activation in ALS, as demonstrated by the finding that microglia activation mediated by extracellular mutant superoxide dismutase 1 was attenuated using TLR2, TLR4 and CD14 blocking antibodies, or when microglia lacked CD14 expression (Zhao et al., 2010). Thus, elevated levels of CD14 in *C9orf72*-mutated microglia could cause increased TLR signaling, with the potential to activate the NLRP3 inflammasome via NF-κB. A role for CD14 in dysregulated TLR activation is also suggested by the present identification of *BGN* as an upregulated gene in *C9orf72*-mutated microglia. The *BGN* gene product, biglycan (BGN), is an ECM-derived damage-associated molecular pattern that acts as a high affinity ligand for CD14 in macrophages and CD14 is required for BGN-mediated TLR2/4-dependent inflammatory signaling pathways in these cells (Roedig et al., 2019). Consistently, BGN can activate the NLRP3 inflammasome in macrophages leading to activation of caspase-1 and release of IL-1β (Babelova et al., 2009). These observations suggest that elevated levels of both BGN and CD14 cause dysregulated NLRP3 inflammasome activation through elevated TLR/NF-κB signaling.

Other ECM/matricellular protein-encoding genes capable of activating TLR/NF-κB-mediated mechanisms, in addition to BGN, were among the DEGs identified in *C9orf72*-mutated microglia in the present study. More specifically, previous studies have shown that the matricellular protein CYR61/CCN1, encoded by DEG *CYR61*, acts as a pattern recognition receptor that binds bacterial pathogen-associated molecular patterns in macrophages, where it interacts directly with TLR2 and TLR4 to activate inflammatory responses (Jun and Lau, 2020). In agreement with this finding, CYR61/CCN1 promotes the expression of NLRP3, ASC,

caspase-1, and IL-1β in astrocytes in an autocrine manner (Jin et al., 2024). In separate investigations, it was shown that knockdown of the *POSTN* gene, encoding periostin (POSTN), a secreted matricellular protein, suppresses caspase-1-mediated pyroptosis and NLRP3 inflammasome activation in muscle cells (Yao et al., 2020). Consistently, POSTN activates inflammation in atopic dermatitis and modulates the immune response and ECM remodeling during atherosclerotic plaque formation (Nunomura et al., 2023; Schwanekamp et al., 2016). Relevant to these observations, POSTN enhances integrin/NF-κB signaling during the process of intervertebral disc degeneration (Zhu et al., 2024). Taken together, these observations suggest that the *C9orf72* HRE causes dysregulation of mechanisms that may underlie an interplay between ECM biology and inflammatory responses in microglia.

Our results also suggest intracellular mechanisms by which the NLRP3 inflammasome may be stimulated through dysregulated activation of TLR/NF-κB signaling. Interleukin 1 Receptor Associated Kinase 4, encoded by DEG *IRAK4*, is a kinase that activates NF-κB downstream of TLR signaling. Mice lacking *IRAK4* display severe impairment of TLR signaling because of the loss of the normal physical association of the cytoplasmic portion of TLRs with IRAK4 (Suzuki et al., 2002). Consistently, inhibition of IRAK4/NF-κB/NLRP3 pathway reduces pyroptosis in hippocampal neurons (Zhao et al. 2024). Thus, it is reasonable to speculate that upregulation of IRAK4 in *C9orf72*-mutated microglia is one of the events contributing to elevated NLRP3 inflammasome, especially if occurring together with other dysregulated mechanisms leading to TLR activation. Another potential mechanism by which the NLRP3 inflammasome could become activated in *C9orf72*-mutated microglia through enhanced TLR signaling is suggested by the present identification of *S100A9* and *S100B* as upregulated genes. Both these genes encode intracellular proteins containing EF-hand calcium-binding motifs. During inflammation, S100A9 and S100B proteins are released in the extracellular milieu where they can bind to TLRs and Receptor for Advanced Glycation End-products (RAGE)

(Franz et al., 2022; Villareal et al., 2014). In microglia, increased level of extracellular S100B upregulates IL-1β and TNF-α expression via RAGE engagement and this effect requires activation of NF-κB (Bianchi et al., 2010). Consistently, extracellular S100B can promote reactive astrocyte transformation leading to a pro-inflammatory phenotype characterized by expression of TLR2 and IL-1β (Villareal et al., 2014). Consistently, extracellular S100A9-stimulates macrophages to undergo NLRP3 inflammasome activation (Sheng et al., 2024). Conversely, ablation of S100A9 in macrophages dampens NLRP3-driven inflammatory response and pyroptosis (Sheng et al., 2023). Of note, *S100A9* expression is downregulated when NLRP3 inflammasome blockade leads to reduced inflammation and ECM remodeling in adipose tissue (Unamuno et al., 2021). Taken together, these observations suggest several mechanisms by which the upregulated genes identified in *C9orf72*-mutated microglia in this study could be involved in NLRP3 inflammasome activation. In this context, an interplay between inflammation and changes at the ECM is suggested by the involvement of several identified DEGs in both these biological processes. Perturbations of this interplay due to dysregulated gene expression is expected to affect not only inflammatory events but also other microglial cell functions, including cell adhesion and cell migration. For instance, DEG *SIGLEC1* encodes a cell adhesion molecule that binds sialylated ligands on endogenous or pathogenic membrane surfaces (Herzog et al, 2022). Inhibition of *SIGLEC1* expression in monocytes reduces levels of proinflammatory cytokines such as IL-1β, IL-6, and IL-8 (Wang et al., 2022). Moreover, SIGLEC1 exerts a proinflammatory effect in chronic obstructive pulmonary disease through activation of NF-κB (Li et al., 2020). These observations identify SIGLEC1 as another cell surface protein potentially involved in pro-inflammatory processes in microglia.

The intrinsic inflammatory phenotype of *C9orf72*-mutated microglia revealed in this study suggested that these cells are toxic to motor neurons. This possibility agrees with the results of

previous studies showing that LPS-primed *C9orf72*-mutated iPSC-derived microglia promote the death of healthy motor neurons in microglia-motor neuron co-culture studies (Banerjee et al., 2023; Vahsen et al., 2023). We examined the possibility that factors released by *C9orf72*-mutated microglia are sufficient to decrease the survival of *C9orf72*-mutated motor neurons in the absence of cell-cell contact. The present studies have shown that exposure to conditioned media from *C9orf72*-mutated microglia, but not isogenic microglia, increases the number of apoptotic *C9orf72*-mutated motor neurons. The effect of the *C9orf72*-mutated microglia conditioned media on isogenic motor neurons was only moderate. This finding provides evidence that *C9orf72*-mutated microglia are primed to be deleterious to motor neurons in a cell-autonomous fashion and that this effect is mediated by factors released by microglia independent of the presence of motor neurons and other neural cells. At least some of the proteins encoded by the *C9orf72*-mutated microglia DEGs identified in this study could contribute to mechanisms of motor neuron degeneration. Both S100A9 and S100B, when secreted by glial cells, can mediate neurotoxic effects. Elevated levels of extracellular S100B are involved in the death of enteric neurons caused by 5-fluorouracil in a RAGE/NF-κB-dependent pathway (Costa et al., 2019). Moreover, acute exposure to extracellular S100B alters the electrophysiological activity of tyrosine hydroxylase-expressing dopaminergic neurons in primary mouse midbrain cultures and S100B overexpression in mice accelerates the loss of substantia nigra pars compacta dopaminergic neurons (Bancroft et al., 2022). S100A9 is pro-inflammatory and highly amyloidogenic in the amyloid-neuroinflammatory cascade in neurodegenerative diseases leading to cognitive impairment (Andrade-Talavera et al., 2022). Consistently, S100A9 inhibition in microglia and macrophages by pharmacological treatment protects against neuronal death *in vitro* and brain injuries *in vivo* in a model of ischemic stroke (Liu et al, 2024).

In conclusion, the transcriptomic changes in *C9orf72*-mutated microglia described in this study provide evidence for a cell-autonomous pro-inflammatory phenotype associated with this mutation. Moreover, they agree with earlier evidence of NLRP3 inflammasome activation in *C9orf72*-mutated microglia and provide previously unavailable information on the identity of several genes that could contribute to inflammasome activation. Some of the proteins encoded by these differentially expressed genes have the potential to participate in both cell-autonomous mechanisms of microglia-mediated inflammation and non-cell autonomous processes of microglia-induced motor neuron degeneration. At least some of the mechanisms mediated by these gene products could provide targets for translational research.

4. Material and Methods

4.1. Human induced pluripotent stem cells

Four previously studied (Chi et al., 2023; Ho et al., 2021; Gao et al., 2024; Thiry et al., 2024) human iPSC lines were used for microglia derivation: the *C9orf72*-mutated iPSC lines CS52iALS-C9n6 (CS52-C9n6-M) and CS29iALS-C9n1 (CS29-C9n1-M), and their matching isogenic control lines CS52iALS-C9n6.ISOxx (CS52-C9n6-ISO) and CS29iALS-C9n1.ISOxx (CS29-C9n1-ISO). These iPSC lines were obtained from Cedars-Sinai (Los Angeles, CA, USA). Undifferentiated state of human iPSCs was assessed by testing for expression of the stem cell markers NANOG and OCT4 using rabbit anti-NANOG (1/1,000; Abcam; Cambridge, UK; Cat. No. ab21624) and rabbit anti-OCT4 (1 µg/ml; Abcam; Cat. No. ab19857) or goat anti-OCT3/4 (1/500; Santa Cruz Biotechnology; Dallas, TX, USA, Cat. No. sc-8628) antibodies, as previously described (Chen et al., 2021).

4.2. Derivation of microglia from human iPSCs

Derivation of microglia from human iPSCs was performed essentially as described in three separate protocols published by Fossati and coworkers (Douvaras et al., 2017), Blurton-Jones and colleagues (McQuade et al., 2018), and Cowley and colleagues (Haenseler et al. 2017). Since we used slightly modified versions of these methods, they are referred to as protocols "P1", "P2" and "P3", respectively. The modifications made to the Douvaras et al. and McQuade et al. methods were previously described (Tang et al., 2022). The following modifications were made to the Haenseler et al. method. Floating progenitor cells were harvested from embryoid bodies weekly and cultured by plating onto 6-well plates or glass coverslips coated with Corning® Matrigel (MilliporeSigma; Burlington, MA, USA; Cat. No. 354277) at 100,000 per cm^2. Cells were cultured for 10-20 days in DMEM/F12 (Thermo-Fisher Scientific; Waltham, MA, USA; Cat. No. 10565-018) with 100 ng/mL IL-34 (Thermo-Fisher Scientific; Cat. No.

200-34), 10 ng/mL GM-CSF (R&D Systems; Minneapolis, MN, USA; Cat. No. 215-GM), N2 supplement (Thermo-Fisher Scientific; Cat. No. 17502-048), 2 mM GlutaMAX (Thermo-Fisher Scientific; Cat. No. 35050-061), and antibiotic-antimycotic (Thermo-Fisher Scientific; Cat. No. 15240-062). All microglia preparations generated using these three protocols were validated through morphological analysis and immunocytochemistry as described (Tang et al., 2022) before being subjected to RNAseq.

4.3. RNA sequencing

Total RNA was isolated for RNAseq from cell pellets obtained from microglia generated with each one of the three protocols described above using sequential treatment with TRIzol Reagent (ThermoFisher Scientific; Cat. No. 15596026) and PureLink RNA Micro Scale Kit (ThermoFisher Scientific; Cat. No. 12183-016) following the instructions provided by the manufacturer. We isolated RNA from iPSC-derived microglia at a series of time points spanning days *in vitro* 45 to 66 for preparations generated with protocols P1 and P2 and days *in vitro* 75 to 108 for preparations induced with protocol P3. These time points were long enough for induced cells to acquire a microglia phenotype according to all three derivation methods (Douvaras et al., 2017; Haenseler et al., 2017; McQuade et al., 2018). All RNA samples were analyzed by Illumina next-generation sequencing at the Genomics platform at the Institute for Research in Immunology and Cancer, Montreal, Quebec, Canada (https://www.iric.ca/en/research/platforms-andinfrastructures/genomics). Adaptor sequences and low-quality bases in the resulting FASTQ files were trimmed using Trimmomatic version 0·35 (Bolger et al., 2014), and genome alignments were conducted using STAR version 2·5 1b (Dobin et al., 2013). Sequences were aligned to the human genome version GRCh38, with gene annotations from Gencode v29 based on Ensemble release 94. As part of quality control, the sequences were aligned to several different genomes to verify that there was no sample

contamination. Raw read-counts were obtained directly from STAR and reads in transcripts per million (TPM) formats were computed using RSEM (Li & Dewey, 2011).

4.4. In silico analysis

To compare isogenic microglia preparations derived with three different protocols, heatmaps showing the levels of expression of 49 microglia/macrophage marker genes (Butovsky et al., 2014; Hickman et al., 2013; Muffat et al., 2016; Zhang et al., 2014) were generated for each sample. Read counts for these specific genes were converted into log2-counts-per-million (logCPM) values as previously described (Tang et al., 2022). Accordingly, negative values represent very low gene expression values. Hierarchical clustering of rows was applied to the heatmaps to group genes with correlated expression patterns, resulting in different gene lists, depending on the samples analyzed in the given heatmap. For each heatmap, color comparisons can be made across samples for each gene as well as between genes for any given sample.

Differential gene expression analysis was conducted on the raw read-count matrix using an edgeR (version 4·0) pipeline as previously described (Chen et al., 2016). Briefly, genes were filtered to retain only those with a robust expression level. A scaling factor of the trimmed mean of M-values (TMM) between each pair of samples was used to normalize the differing RNA composition of each sample. The differentiation protocol and experimental batch were incorporated into the design used for differential expression analysis since they were the two known sources of variability in the studied samples. The “glmQLFit” and “glmQLFTest” functions of edgeR were then used to obtain DEGs with the quasi-likelihood (QL) method. Genes were considered statistically significant if the adjusted p-value (false discovery rate; FDR) was <0.05, with no fold-change cutoff applied to restrict the number of DEGs identified.

Gene set enrichment analysis (GSEA) was conducted on the DEGs using the Functional Annotation Tool of DAVID Bioinformatics Resources 6·8 (Huang da et al., 2009a; 2009b). GO

terms and KEGG pathways enriched in the DEGs were computed separately for upregulated and downregulated genes, as this increases the statistical power to identify pathways/terms pertinent to the phenotype of interest (Hong et al., 2013). For each GO category (e.g., Biological Process or Molecular Function), the "direct" function in DAVID was used to restrict the results to those directly annotated by the source database and to avoid repetitive and generic parent terms. For the KEGG pathways, only the non-disease-specific pathways are shown. For both GO and KEGG pathways analysis, all terms with associated FRD < 1 are shown, except for categories presenting more than 10 terms, in which case only the more statistically significant terms with associated FDR<e^{-6} are shown.

4.5. Open-source data

Raw read counts from RNA sequencing of undifferentiated human iPSCs were obtained from the LINCS Data Portal (LSC-1002 and LSC-1004 from Dataset LDS-1355) (http://lincsportal.ccs.miami.edu/datasets/). LSC-1002 and LSC-1004 correspond to human iPSC lines CS14i-CTR-n6 and CS25iCTR-18n2, provided by Cedar Sinai Stem Cell Core Laboratory. These raw data were combined with the RNAseq data from the present microglia preparations and analyzed using a single pipeline, to generate heatmaps comparing the levels of expression of activated microglia marker genes in each sample.

4.6. Incubation of cultures of iPSC-derived motor neurons with microglia conditioned media

Motor neuron cultures enriched in phrenic motor neurons were derived from human iPSC lines CS29-C9n1-M and its matching isogenic control line CS29-C9n1-ISO according to a previously described protocol (Thiry et al., 2024). Briefly, neural progenitor cells were dissociated on day 6 and split 1:5 with a chemically defined neural medium including DMEM/F12 supplemented with GlutaMAX (1/1; Thermo-Fisher Scientific; Cat. No. 35050-

061), Neurobasal medium (1/1; Thermo-Fisher Scientific; Cat. No. 21103-049), N2 (0.5X; Thermo-Fisher Scientific; Cat. No. 17504-044), B27 (0.5X; Thermo-Fisher Scientific; Cat. No. 17502-048), ascorbic acid (100 µM; Sigma-Aldrich; Oakville, ON, Canada; Cat. No. A5960), antibiotic-antimycotic (1X; Thermo-Fisher Scientific; Cat. No. 15240-062), supplemented with retinoic acid (RA) (1 µM, Sigma-Aldrich; Cat. No. R2625) and purmorphamine (0.125 µM, Sigma-Aldrich; Cat. No. SML-0868) in combination with 1 µM CHIR99021 (STEMCELL Technologies; Vancouver, BC, Canada; Cat. No. 72054), 2 µM DMH1 (Sigma-Aldrich; Cat. No. D8946) and 2 µM SB431542 (Tocris Bioscience; Bristol, UK; Cat. No. 1614). The culture medium was changed every other day for 6 days and the resulting motor neuron progenitors were then expanded for 6 days with the same medium containing 3 µM CHIR99021, 2 µM DMH1, 2 µM SB431542, 1 µM RA, 0.125 µM purmorphamine and 500 µM valproic acid (Sigma-Aldrich; Cat. No. P4543). Motor neuron progenitors were dissociated and plated at 50,000 cells/well on coverslips coated with Dendritic Polyglycerol Amine/Matrigel substrate as described previously (Thiry et al., 2022). Motor neurons were differentiated in the same neural medium supplemented with 1 µM RA and 0.125 µM purmorphamine, 0.1 µM Compound E (MilliporeSigma; Cat. No. 565790), insulin-like growth factor 1 (10 ng/mL; R&D Systems; Cat. No. 291-G1-200), brain-derived neurotrophic factor (10 ng/mL; Thermo-Fisher Scientific; Cat. No. PHC7074) and ciliary neurotrophic factor (10 ng/mL; R&D Systems; Cat. No. 257-NT-050). Motor neuron culture medium was replaced every other day for one week. Motor neuron cultures were validated by immunocytochemistry and single-cell RNA sequencing as described (Thiry et al., 2020; 2022; 2024). For studies with microglia conditioned media, after one week in motor neuron culture medium, the medium was replaced every other day for an additional 2-weeks with a mixture of motor neuron culture medium (50%) and microglia conditioned medium (50%) obtained from either *C9orf72*-mutated or isogenic microglia derived from CS29-C9n1-M or CS29-C9n1-ISO iPSCs using protocol P2, as

described above. After 46 days of culture (3-weeks post-plating), culture media were removed abd motor neurons were rinsed and analyzed by immunocytochemistry.

4.7. Analysis of apoptosis in iPSC-derived motor neuron cultures

Immunocytochemistry with rabbit anti-Cleaved-Caspase 3 (CC3 (Asp175); 1/400; Cell Signaling; New England Biolabs, Ltd., Ontario, Canada; Cat. No. 9661S) in combination with goat anti-CHAT antibody (1/100; Millipore; Cat. No. MAB144P) was performed to detect apoptotic motor neurons in cultures obtained from CS29-C9n1-M or CS29-C9n1-ISO iPSCs, in the absence or presence of conditioned media from CS29-C9n1-M or CS29-C9n1-ISO microglia. For quantification, images in 3 random fields were taken with a 20X objective using an Axio Observer Z1 microscope connected to an AxioCam camera using ZEN software (Zeiss) and analyzed with Image J.

4.8. Statistical analysis of motor neuron cell death assays

To account for culture variability and ensure experimental reproducibility, studies with motor neurons plus or minus microglia conditioned media were performed with at least 3 biologically independent cultures per condition. Error bars shown are means ± standard error of means (SEM) of the average. Two-way repeated measure ANOVA and Tukey's multiple comparisons post-hoc test were used to detect differences in the proportion of CC3-positive motor neurons in isogenic and *C9orf72*-mutated-derived cells in each of the three culture conditions tested, namely absence of microglia conditioned media, presence of isogenic microglia conditioned media, or presence of *C9orf72*-mutated microglia conditioned media.

Author Contributions

N.S.P. and L.T. performed bioinformatics studies. Y.T. performed all tissue culture and microscopy studies. L.T. performed studies with motor neurons. S.S. supervised the study. All authors have read and agreed to the uploaded version of the manuscript.

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Competing Interests

The authors declare that there is no conflict of interest.

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Data Sharing Statement: Read counts of the RNAseq data and the R code used for *in silico* analyses will be made available.

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