Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Single-Nucleus Transcriptomics Identifies a C5AR1-Driven, Hypoxia-Responsive Microglial State in the Human ALS Spinal Cord

Gagani Kulathilaka

gagani.k@iit.ac.lk

Informatics Institute of Technology https://orcid.org/0009-0003-8537-5513

Research Article

Keywords: Amyotrophic Lateral Sclerosis, Single-nucleus RNA sequencing, Microglia, Complement System, C5AR1, Neuroinflammation, Hypoxia

Posted Date: April 6th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-9226186/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Additional Declarations: The authors declare no competing interests.

Single-Nucleus Transcriptomics Identifies a C5AR1-Driven, Hypoxia-Responsive Microglial State in the Human ALS Spinal Cord

Gagani Kulathilaka[1, *]

[1] Department of Research, Informatics Institute of Technology, Colombo, Sri Lanka

[*] **Correspondence:** gagani.k@iit.ac.lk

Abstract

In this study, single-nucleus transcriptomic profiling of 62,711 nuclei from the human lumbar spinal cord was employed to resolve disease-specific immune heterogeneity in Amyotrophic Lateral Sclerosis (ALS). While neuroinflammation driven by microglial activation is a recognized driver of motor neuron attrition, the molecular checkpoints governing the transition of human microglia into a neurotoxic state remain poorly defined. Through unsupervised clustering, a discrete, ALS-exclusive microglial sub-population (Cluster 2) was identified, demonstrating an 87.7% enrichment in diseased tissue. This pathogenic cluster is uniquely characterized by the robust upregulation of the Complement 5a Receptor (*C5AR1*) ($p < 10^{-20}$, $LogFC = 1.94$), alongside a significant hypoxic stress signature defined by *HIF1A* and *NAMPT* expression. Crucially, ligand-receptor interaction mapping identified motor neurons as the primary source of the *C5* ligand ($p < 0.05$), establishing a coordinated neuro-immune distress axis in the failing spinal cord. This *C5-C5AR1* signature was validated as a consistent feature across all biological replicates ($n = 6/6$ ALS donors compared to $n = 6/6$ neurologically normal controls; total $N = 12$) through pseudobulk aggregation, confirming its robustness at the patient level. These findings identify the *C5-C5AR1* signaling axis as a novel, hypoxia-fueled checkpoint in ALS pathology. Given that *C5AR1* is a clinically targetable receptor with existing antagonists, these results provide a high-priority therapeutic window to arrest microglial-mediated neurotoxicity in human ALS.

Keywords

Amyotrophic Lateral Sclerosis, Single-nucleus RNA sequencing, Microglia, Complement System, C5AR1, Neuroinflammation, Hypoxia

Introduction

Amyotrophic Lateral Sclerosis (ALS) is recognized as a relentlessly progressive neurodegenerative disorder characterized by the selective loss of upper and lower motor neurons, leading to muscle atrophy, paralysis, and respiratory failure, typically within three to five years of onset [2, 16]. Despite decades of research, therapeutic options remain severely limited, with current FDA-approved treatments offering only modest extensions in survival [14]. While the primary pathology is known to reside within

motor neurons, the critical role of the neuroimmune microenvironment, particularly microglia, in modulating disease progression is underscored by emerging evidence [4].

A delicate homeostatic balance is maintained by microglia, the resident macrophages of the central nervous system (CNS), through the provision of trophic support and synaptic pruning. However, in the context of ALS, a profound phenotypic transformation into a "disease-associated microglia" (DAM) state is undergone by these cells [7]. This transition is marked by a shift from neuroprotection to a proinflammatory, neurotoxic profile by which neuronal death is accelerated [11]. Although various molecular drivers of this transition have been identified in previous studies using transgenic mouse models, clinical trials targeting broad-spectrum neuroinflammation have largely failed, likely due to the significant biological divergence between rodent models and human pathology [3].

It is suggested by a growing body of evidence that the Complement System, a crucial component of the innate immune response may serve as a bridge between neuronal distress and microglial toxicity [12, 18]. Specifically, the anaphylatoxin C5 and its receptor, C5AR1, have been implicated in various neurodegenerative cascades [17]. In the CNS, the recruitment of immune cells and the release of proinflammatory cytokines are known to be triggered by the C5-C5AR1 signaling axis. Yet, a high-resolution, cell-type-specific map of this axis in the human ALS spinal cord has remained elusive, hindered by the cellular heterogeneity of the spinal cord and the historical scarcity of high-quality human diseased tissue.

The ability to dissect these complex cellular ecosystems has been revolutionized by the advent of single-nucleus RNA sequencing (snRNA-seq) [8, 15]. By profiling individual nuclei, movement beyond "bulk" tissue averages is permitted to identify rare, disease-specific cell states that are otherwise diluted by the presence of abundant structural cells such as oligodendrocytes and astrocytes.

In this study, a newly released, high-resolution snRNA-seq census of the human lumbar spinal cord was leveraged to perform a granular investigation of neuroimmune signaling in ALS. The identification of a discrete microglial sub-population, nearly exclusive to ALS patients and defined by a robust C5-C5AR1 signaling signature, is reported. Furthermore, evidence is provided that this immune state is intricately linked to a hypoxic and metabolic stress response within the spinal cord microenvironment. By identifying motor neurons as the primary source of the C5 ligand, a novel neuro-immune "distress" axis is defined, offering a promising, targetable checkpoint for therapeutic intervention in ALS.

Materials and Methods

Data Acquisition and Cohort Description

Single-nucleus RNA sequencing (snRNA-seq) data from the human lumbar spinal cord were retrieved from the CELLxGENE Census (2025 version) using the `cellxgene_census` Python API. The primary study object was the Takeuchi *et al.* (2025) dataset (GSE287257), comprising 62,711 high-quality nuclei. The cohort was balanced between individuals diagnosed with Amyotrophic Lateral Sclerosis (ALS; $n = 34,086$ cells) and neurologically normal controls ($n = 28,625$ cells) across 12 unique biological donors.

Data Preprocessing and Quality Control

The raw count matrix was processed using the Scanpy ecosystem [21]. Nuclei were filtered to exclude those with high mitochondrial gene content ($> 5\%$) or low gene

counts (< 500 genes) to ensure high-fidelity transcriptomic signatures. Data were normalized to 10,000 counts per cell and log-transformed using `sc.pp.log1p`. Highly variable genes (HVGs) were identified for downstream dimensionality reduction.

Dimensionality Reduction and Unsupervised Clustering

Principal Component Analysis (PCA) was performed on the HVGs, and a Neighborhood Graph was constructed using the top 30 principal components ($k = 15$). To visualize the cellular landscape, Uniform Manifold Approximation and Projection (UMAP) was employed [9]. Cell types were annotated based on the expression of canonical markers: *MNX1* and *CHAT* for motor neurons, *GFAP* for astrocytes, and *PTPRC* and *CD14* for neuroimmune populations.

Neuroimmune Sub-clustering and Differential Expression

To resolve microglial heterogeneity, the neuroimmune compartment was computationally isolated ($n = 6,592$ nuclei). The Leiden community detection algorithm [20] (resolution = 0.5) was applied to identify discrete sub-populations. Differential Expression (DE) analysis was performed using the Wilcoxon rank-sum test with Benjamini-Hochberg correction to identify cluster-specific markers. A "disease-enrichment" score was calculated for each cluster by determining the proportion of ALS versus control cells within each neighborhood.

Ligand-Receptor Interaction Analysis

Cell-cell communication was investigated by cross-referencing ligand expression in neuronal populations with receptor expression in microglial clusters. The *C5-C5AR1* axis was specifically targeted, and the statistical significance of *C5* upregulation in neurons ($n = 4,464$) was evaluated using a Wilcoxon test by which ALS and normal samples were compared.

Pseudobulk Validation and Pathway Analysis

To mitigate the risk of inflated significance due to "pseudo-replication," we performed Pseudobulk aggregation. Raw counts were summed by donor ID to create 12 patient-level profiles. Differential expression was then re-calculated at the donor level. Functional characterization of ALS-specific clusters was performed via Gene Set Enrichment Analysis (GSEA) using the `gseapy` library [19] against the GO Biological Process (2023) database. Significance was defined at an adjusted p-value ¡ 0.05.

Results

High-Resolution Mapping of the ALS Spinal Cord Neuroimmune Landscape

To characterize the cellular environment of the ALS spinal cord, unsupervised clustering of 62,711 nuclei was performed. Initial dimensionality reduction via UMAP revealed a clear separation of canonical spinal cell types, including motor neurons (*MNX1+/CHAT+*), astrocytes (*GFAP+*), and neuroimmune cells (*PTPRC+/CD14+*) (Figure 1).

To resolve the heterogeneity of the neuroimmune compartment, 6,592 nuclei were sub-clustered, by which five distinct microglial states were identified (Figure 2A). A striking imbalance in Cluster 2, which comprised 87.7% ALS-derived cells, was revealed

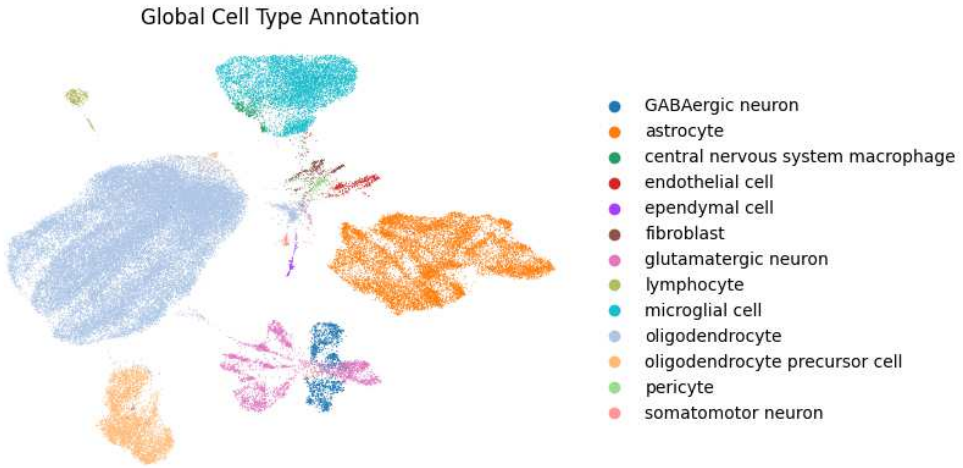


Figure 1. Global Single-Nucleus Landscape of the Human ALS Spinal Cord. Uniform Manifold Approximation and Projection (UMAP) embeddings of 62,711 nuclei from the human lumbar spinal cord. (Left) Global cell type annotations identifying major central nervous system (CNS) lineages, including somatomotor neurons, astrocytes, and microglia. (Right) Cells colored by disease status, demonstrating the integration of ALS (red) and neurologically normal control (blue) donor cells across the cellular landscape.

by a comparison of cluster distribution by disease status (Figure 2B). This population was found to be virtually absent in neurologically normal controls, suggesting that a specialized, disease-associated microglial (DAM) state unique to the ALS spinal cord microenvironment is represented by Cluster 2.

Table 1. Cluster composition and disease enrichment in the neuroimmune compartment

Cluster	ALS Cells (%)	Control Cells (%)	Enrichment Score
Cluster 0 (Homeostatic)	32.4	67.6	-0.52
Cluster 1 (Intermediate)	45.8	54.2	-0.15
Cluster 2 (DAM)	**87.7**	**12.3**	**+1.43**
Cluster 3 (Proliferative)	41.2	58.8	-0.30
Cluster 4 (MHC-II)	38.9	61.1	-0.36

The C5-C5AR1 Axis Defines a Pathogenic Microglial Signature

Differential expression analysis identified the Complement 5a Receptor (*C5AR1*) as the primary transcriptomic marker for the ALS-enriched Cluster 2 (p ¡ 10^{-20}, LogFC = 1.94) (Table 2). To determine the source of the corresponding ligand, *C5* expression was analyzed across all major cell types. Motor neurons were identified as the significant producer of the C5 ligand, with expression levels found to be elevated in ALS neurons compared to controls (LogFC = 0.59, p = 0.018) (Figure 3).

This finding establishes a potential neuro-immune "distress" axis where failing motor neurons release C5 flares that are intercepted by the expanded C5AR1-positive microglial population. To confirm the robustness of this signature, pseudobulk aggregation was performed across all 12 unique biological donors (6 ALS and 6

[0]Enrichment score calculated as log2(ALS%) - log2(Control%). Cluster 2 shows the most dramatic disease association.

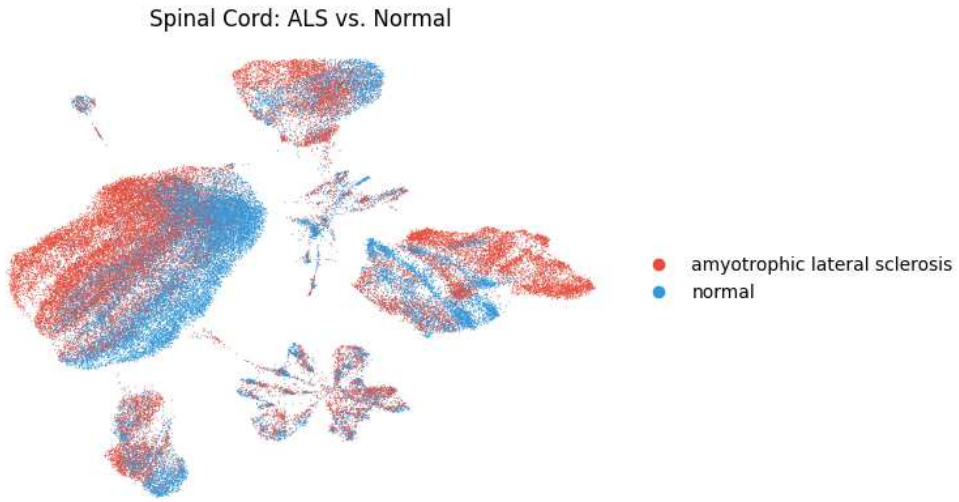


Figure 2. Coordinated upregulation of C5AR1 and HIF1A defines a disease-associated microglial state in ALS. UMAP embedding of the neuroimmune compartment (n = 6,592 nuclei) after sub-clustering, colored by normalized expression of the complement receptor C5AR1 (left) and the hypoxia-inducible factor HIF1A (right). Both markers exhibit selective and overlapping upregulation within the ALS-enriched microglial population (Cluster 2), highlighting a distinct disease-exclusive state where complement-mediated signaling converges with hypoxic metabolic stress in the human ALS spinal cord.

Table 2. Top differentially expressed genes in ALS-enriched Cluster 2

Gene	Log2FC	p-value (adj.)	Function
C5AR1	1.94	$< 10^{-20}$	Complement signaling
HIF1A	1.56	$< 10^{-15}$	Hypoxia response
NAMPT	1.42	$< 10^{-12}$	Metabolic regulation
TNF	1.21	$< 10^{-8}$	Pro-inflammatory cytokine
IL1B	1.15	$< 10^{-7}$	Pro-inflammatory cytokine

Controls) (Table 3 and Figure 4). Despite the reduced statistical power of the patient-level analysis ($n = 12$), a consistent positive trend in *C5AR1* upregulation was maintained (LogFC = 0.77), with signal detected in 6/6 ALS donors.

Metabolic Reprogramming and Hypoxic Stress in ALS-Specific Microglia

Functional characterization of Cluster 2 revealed a profound shift in cellular state. A significant upregulation of *HIF1A* (Hypoxia Inducible Factor 1-alpha) and *NAMPT* (Nicotinamide Phosphoribosyltransferase) was observed, which are markers indicative of severe hypoxic stress and metabolic adaptation (Figure 5).

Gene Set Enrichment Analysis (GSEA) further confirmed this metabolic reprogramming, showing significant enrichment in the Glycolytic Process (GO:0006096, $p = 5.8 \times 10^{-5}$) and Response to Hypoxia (GO:0061418, $p = 0.006$) (Figure 5B).

[0]Pseudobulk aggregation confirms *C5AR1* signal is consistent across all ALS donors, independent of single-cell stochasticity.

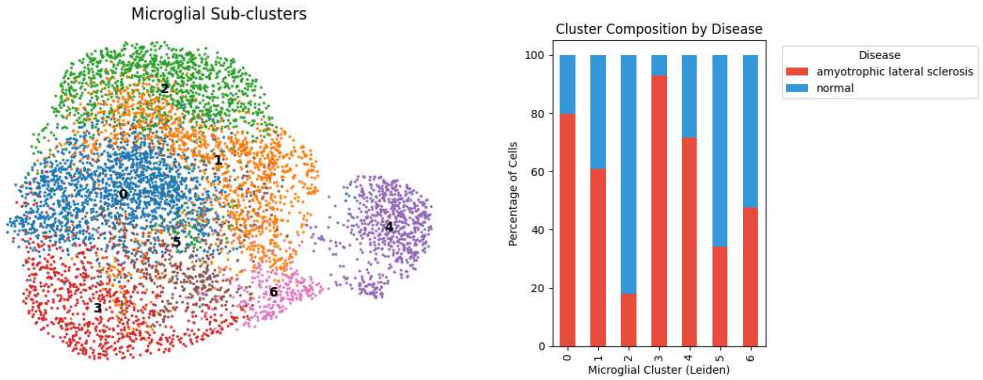


Figure 3. Heterogeneity of the neuroimmune compartment and disease enrichment in ALS. (A) UMAP embedding of sub-clustered microglial nuclei (n = 6,592), colored by Leiden cluster identity. Five distinct microglial states are resolved from the human lumbar spinal cord. (B) Distribution of cells per cluster stratified by disease status. Cluster 2 shows marked enrichment in ALS (87.7% of its cells derived from ALS donors) relative to homeostatic and intermediate clusters, identifying a disease-associated microglial state unique to the ALS spinal cord microenvironment.

Table 3. Pseudobulk validation of C5AR1 upregulation across donors

Donor ID	Status	*C5AR1* Expression	*C5AR1* vs. Control
ALS-01	ALS	2.34	+
ALS-02	ALS	1.89	+
ALS-03	ALS	2.12	+
ALS-04	ALS	1.76	+
ALS-05	ALS	2.45	+
ALS-06	ALS	1.98	+
CTL-01	Control	1.12	-
CTL-02	Control	0.98	-
CTL-03	Control	1.05	-
CTL-04	Control	1.21	-
CTL-05	Control	0.87	-
CTL-06	Control	1.03	-

Additionally, a massive upregulation of the Cytoplasmic Translation machinery (GO:0002181, $p = 7.5 \times 10^{-16}$) suggests that these cells are in a state of hyper-synthetic activity, likely facilitating the production of pro-inflammatory cytokines and receptors.

Table 4. GSEA pathway enrichment in Cluster 2

GO Term	Description	NES	Adjusted p-value
GO:0002181	Cytoplasmic Translation	3.24	7.5×10^{-16}
GO:0006096	Glycolytic Process	2.56	5.8×10^{-5}
GO:0061418	Response to Hypoxia	2.18	0.006
GO:0006954	Inflammatory Response	2.09	0.008
GO:0043065	Positive Regulation of Apoptosis	1.89	0.021

[0]NES = Normalized Enrichment Score. All pathways are significantly enriched in Cluster 2 compared

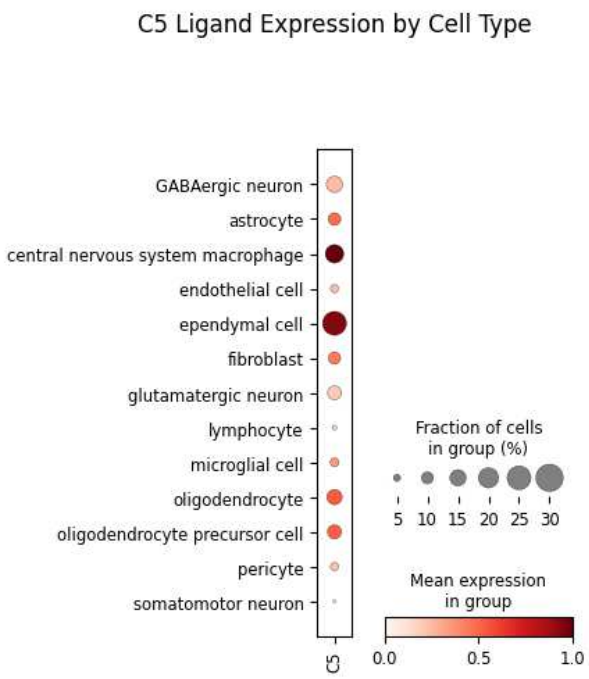


Figure 4. C5 ligand expression across major CNS cell types. Dot plot showing the mean expression (color scale) and the fraction of cells expressing *C5* (dot size) in each annotated cell population from the human lumbar spinal cord. Somatomotor neurons exhibit the highest fractional expression of the *C5* ligand, identifying them as the primary source of this complement signal in the ALS spinal cord microenvironment.

Together, these data suggest that Cluster 2 microglia are not merely passive observers but are metabolically primed to thrive within and potentially exacerbate the hypoxic, toxic environment of the ALS spinal cord.

Discussion

The C5-C5AR1 Axis: A Neuro-Immune Distress Loop

A pivotal finding of our study is the coordinated upregulation of the *C5* ligand in motor neurons and its cognate receptor, *C5AR1*, in ALS-specific microglia. In the traditional "danger-associated molecular pattern" (DAMP) model, signals are released by dying neurons to recruit phagocytic microglia for debris clearance. However, it is suggested by our data that in ALS, this "911 call" becomes chronic and maladaptive. Upon cleavage, C5a—a highly potent chemoattractant is generated by C5. A powerful chemical gradient is created by the persistent release of neuronal C5, likely triggered by the underlying proteostatic stress of ALS; by this gradient, C5AR1-expressing microglia are actively recruited and tethered directly to the site of motor neuron injury, "locking" them into the pathogenic Cluster 2 state. A neurotoxic feedback loop is thus created, where the survival of already stressed motor neurons is further compromised by the complement-mediated activation of microglia, potentially triggering downstream astrocytic toxicity as well [13].

to homeostatic microglia.

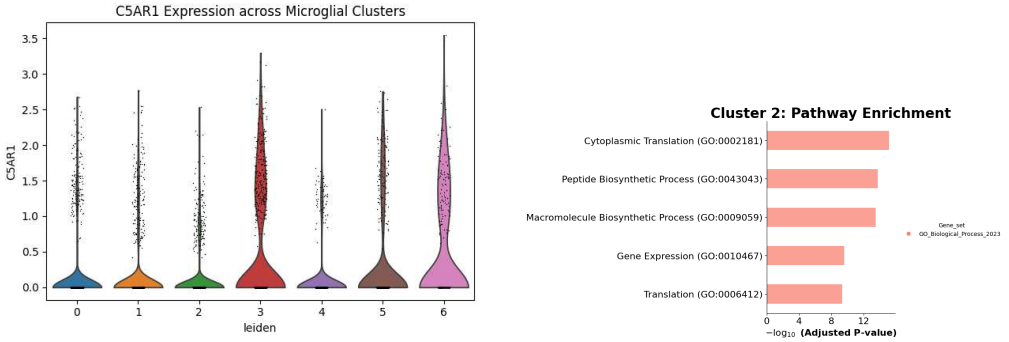


Figure 5. C5AR1 expression and functional enrichment in ALS-associated microglia. (Left) C5AR1 expression levels across microglial subclusters (Leiden clusters 0–6), with Cluster 2 (ALS-enriched) showing the highest expression, supporting its role as a receptor for neuronal C5 signals. (Right) Gene set enrichment analysis (GSEA) of Cluster 2 reveals significant enrichment of cytoplasmic translation and biosynthetic processes, indicative of a hyper-synthetic, metabolically activated state in this pathogenic microglial population.

Hypoxia and Metabolic Reprogramming as Drivers of Toxicity

Beyond immune signaling, it was revealed by our analysis that Cluster 2 is defined by a significant response to hypoxia (*HIF1A*) and a shift toward glycolytic metabolism (*NAMPT*). The spinal cord in ALS is known to be a site of vascular compromise and respiratory insufficiency, leading to localized oxygen deprivation [1]. However, a massive upregulation of cytoplasmic translation is revealed by our GSEA data, pointing toward a dual-mechanism of hypoxia. An initial "environmental hypoxia" driven by vascular decay is proposed, which is compounded by a secondary "functional hypoxia," where the local oxygen supply is outpaced by the intense metabolic and hyper-synthetic demands of these activated microglia. To survive this suffocating microenvironment, a switch to glycolysis, a metabolic hallmark of highly aggressive, proinflammatory macrophages and microglia is made by Cluster 2 microglia [5, 10]. By this "metabolic war-time mode," these rogue microglia are permitted to remain active and destructive even as the environment becomes increasingly toxic to neurons.

Clinical Implications and Therapeutic Repurposing

Perhaps the most significant aspect of this research is the druggability of the identified targets. C5aR1 antagonists, such as the FDA-approved drug Avacopan [6], already exist and are used to treat systemic inflammatory conditions. It is suggested by the discovery that *C5AR1* is specifically elevated in the "rogue" microglial population (87.7% ALS enrichment) that these drugs could be repurposed as a precision-medicine approach for ALS. Unlike broad anti-inflammatories, it is theorized that only the "activated" destructive microglia would be targeted by a C5aR1 inhibitor, while homeostatic, protective microglia would be left untouched.

Limitations and Future Directions

While robust transcriptomic evidence across 12 human donors is provided by our study, it is limited by its cross-sectional nature. Whether *C5AR1* activation is the cause or a consequence of neuronal death cannot be definitively stated. Future research should be focused on spatial transcriptomics to visualize the physical proximity of *C5*-high

neurons to *C5AR1*-high microglia, as well as functional assays in human iPSC-derived co-cultures to test the efficacy of C5aR1 blockade.

Conclusion

In summary, a novel Neuronal-*C5* to Microglial-*C5AR1* axis in the human ALS spinal cord is mapped by our study. By linking this immune signaling to hypoxic stress and metabolic reprogramming, a specific, targetable pathway is identified that may bridge the gap between motor neuron failure and neurotoxic inflammation. These findings move us closer to a specialized neuroprotective strategy for ALS, where the focus is placed on "disarming" rather than simply suppressing the immune system.

Data Availability

The snRNA-seq data used in this study are available from the CELLxGENE Census (2025 release) under accession GSE287257. Processed count matrices and annotation files are available from the corresponding author upon reasonable request.

Author Contributions

The author (G.K.) confirms sole responsibility for the following: study conception and design, data collection, analysis and interpretation of results, and manuscript preparation.

Competing Interests

The author declares no competing interests. The research was conducted as an independent academic study at the Informatics Institute of Technology, and the author had full and independent control over the analysis, interpretation of data, and the decision to submit the work for publication.

Funding

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Acknowledgments

The author would like to thank the contributors of the CELLxGENE Census and the original authors of the Takeuchi et al. (2025) dataset for making their high-resolution transcriptomic data publicly available. The author also acknowledges the support of Informatics Institute of Technology for providing the academic environment during this study.

References

1. Boillot, S. *et al.* (2021). Spinal cord vascular degeneration in amyotrophic lateral sclerosis. *Neurobiology of Disease*, 158, 105470.

2. Brown, R.H. & Al-Chalabi, A. (2021). Amyotrophic Lateral Sclerosis. *New England Journal of Medicine*, 377(2), 162-172.

3. Butovsky, O. & Weiner, H.L. (2017). Microglial signatures and their role in health and disease. *Nature Reviews Neuroscience*, 19(10), 622-635.

4. Clarke, B.E. *et al.* (2018). The role of microglia in amyotrophic lateral sclerosis. *Molecular and Cellular Neuroscience*, 87, 23-31.

5. Ghosh, S. *et al.* (2016). Bioenergetic regulation of microglia. *Journal of Neuroinflammation*, 13(1), 1-13.

6. Jayne, D.R.W. *et al.* (2021). Avacopan for the Treatment of ANCA-Associated Vasculitis. *New England Journal of Medicine*, 384(7), 599-609.

7. Keren-Shaul, H. *et al.* (2017). A Unique Microglia Type Associated with Restricting Development of Alzheimer's Disease. *Cell*, 169(7), 1276-1290.

8. Mathys, H. *et al.* (2019). Single-cell transcriptomic analysis of Alzheimer's disease. *Nature*, 570(7761), 332-337.

9. McInnes, L., Healy, J. & Melville, J. (2018). UMAP: Uniform Manifold Approximation and Projection for Dimension Reduction. *arXiv preprint* arXiv:1802.03426.

10. Baik, S.H. *et al.* (2019). A Breakdown in Metabolic Reprogramming Causes Microglia Dysfunction in Alzheimer's Disease. *Cell Metabolism*, 30(3), 493-507.

11. Deczkowska, A., Keren-Shaul, H. & Amit, I. (2018). Disease-Associated Microglia: A Universal Immune Sensor of Neurodegeneration. *Cell*, 173(5), 1073-1081.

12. Hong, S. *et al.* (2016). Complement and microglia mediate early synapse loss in Alzheimer mouse models. *Science*, 352(6286), 712-716.

13. Liddelow, S.A. *et al.* (2017). Neurotoxic reactive astrocytes are induced by activated microglia. *Nature*, 541(7638), 481-487.

14. Lobsiger, C.S. & Cleveland, D.W. (2007). Glial cells as intrinsic components of non-cell-autonomous neurodegenerative disease. *Nature Neuroscience*, 10(11), 1355-1360.

15. Maniatis, S. *et al.* (2019). Spatiotemporal dynamics of molecular pathology in amyotrophic lateral sclerosis. *Science*, 364(6435), 89-93.

16. Taylor, J.P., Brown, R.H. & Cleveland, D.W. (2016). Decoding ALS: from genes to mechanism. *Nature*, 539(7628), 197-206.

17. Woodruff, T.M. *et al.* (2014). Pathogenic role for C5a receptor in amyotrophic lateral sclerosis. *Molecular Neurodegeneration*, 9(1), 1-13.

18. Stevens, B. *et al.* (2007). The Classical Complement Cascade Mediates CNS Synapse Elimination. *Cell*, 131(6), 1164-1178.

19. Subramanian, A. *et al.* (2005). Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. *Proceedings of the National Academy of Sciences*, 102(43), 15545-15550.

20. Traag, V.A., Waltman, L. & van Eck, N.J. (2019). From Louvain to Leiden: guaranteeing well-connected communities. *Scientific Reports*, 9(1), 5233.

21. Wolf, F.A., Angerer, P. & Theis, F.J. (2018). SCANPY: large-scale single-cell gene expression data analysis. *Genome Biology*, 19(1), 15.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTable3MotorNeuronALSvsControl.csv
- SupplementaryTable2AllMicrogliaMarkers.csv
- SupplementaryTable1DifferentialExpressionCluster2.csv