Investigating the role of microglial Interleukin 6 (IL-6) in a mouse model of amyotrophic lateral sclerosis.

Abstract

Amyotrophic lateral sclerosis (ALS) is a fatal and incurable neurodegenerative disorder characterized by progressive motor neuron degeneration, leading to muscle weakness, paralysis, respiratory failure, and death. The global incidence of ALS is 1-2.6 cases per 100,000 persons, with a prevalence of about 6 cases per 100,000. Neuroinflammation, particularly involving microglia and the cytokine interleukin-6 (IL-6), has been implicated in the pathogenesis of the disease. This research aims to investigate the role of microglial IL-6 in ALS progression using the SOD1^G93A mouse model, with the hypothesis that modulation of microglial IL-6 levels influences motor neuron degeneration. First, microglial IL-6 expression is assessed at different disease stages to establish its baseline levels and evaluate its correlation to disease progression. Next, the levels of microglial IL-6 are modulated to investigate the impact of its downregulation and upregulation on neuroinflammatory markers, motor neuron counts and disease progression, assessed by immunohistochemistry and motor performance evaluation. The IL-6 levels modulation uses capsid-modified recombinant adeno-associated virus type 6 (rAAV6) to transduce microglia and increase or silence the IL-6 gene expression. The neuroinflammatory markers monitored are microglia and astrocyte activation. Finally, the therapeutic potential of targeting IL-6 signalling in microglia is evaluated, using anti-IL6 (siltuximab) and anti-IL-6 receptor (tocilizumab) agents encapsulated in PLGA nanoparticles and administered intravenously to the control, early- and late-disease groups, so that its impact on neuroinflammation and disease progression can be assessed.

Lay Abstract

Amyotrophic lateral sclerosis (ALS) is an incurable disease affecting certain cells – called motor neurons – in brain and spinal cord, causing gradually increasing muscle weakness, eventually resulting in inability to move, swallow and breathe. Every year, around 2 out of 100,000 people develop the disease, leaving them with approximately 2-5 years to live. While it is still not known exactly how the disease begins, evidence suggests that inflammation in the brain and spinal cord is involved. Particularly important seem to be brain defence units, microglia, and a cell signalling substance they produce, called interleukin-6 (IL-6). This research aims to understand, using a specific mouse model of the disease, what the role of IL-6 in the course of ALS is, with the assumption that altering its levels will influence the extent to which ALS damages motor neurons. First, the amount of microglial IL-6 will be measured in mice at different stages of the disease to assess how IL-6 relates to the disease progression. In the next step, the levels of IL-6 will be increased and decreased, using microglial gene manipulation, and the effect on inflammation and condition of the animals will be evaluated. Eventually, medicine will be used to stop IL-6 from attaching to its target or to block IL-6 itself, and the results on the disease progression and inflammation will be assessed.

Background

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder characterized by progressive degeneration of both upper and lower motor neurons, leading to weakness and paralysis of voluntary muscles, including inspiratory, expiratory and upper airway muscles, ultimately resulting into respiratory failure and death (Nichols et al., 2013). Globally, ALS

occurs with an annual incidence of approximately 1-2.6 cases / 100 000 persons, and the prevalence is approximately 6 cases / 100 000 persons (Talbott et al., 2016).

The average age of ALS onset is between 50-60 years of age, and the disease usually follows a rapidly progressive course with a median survival time of 2-4 years (Kjældgaard et al., 2021). Currently it is known that ALS shares features of frontotemporal dementia (FTD), and nearly 50 % of all patients suffer from varying degrees of cognitive and behavioural impairment (Abramzon et al., 2020).

The vast majority of ALS cases (approximately 90 %) have no known aetiology and are termed “sporadic”, as opposed to familial ALS cases resulting from a number of gene mutations (Nichols et al., 2013).

While the exact pathogenesis of ALS is still unknown and multiple pathways seem to be involved, analysis of cerebrospinal fluid (CSF) and postmortem spinal cord samples demonstrated an increase in microglial activation and lymphocyte permeation, suggesting that neuroinflammation plays a role (Liu & Wang, 2017). Further research revealed astrocyte activation, T lymphocyte infiltration and overproduction of inflammatory cytokines – all characterizing neuroinflammation – occurring together with neuronal loss (Endo et al., 2016).

Amongst the several cytokines reported to have increased expression in ALS, interleukin-6 (IL-6) has been implicated to play a central role (Tortelli et al., 2020). IL-6 is a multifunctional cytokine that influences various cellular functions, has both anti-inflammatory and pro-inflammatory properties and plays an important role in regulating inflammatory processes in the CNS (Scheller et al., 2011).

Il-6 was found to be markedly increased in ALS (as well as FTD) patients as compared to controls (Galimberti et al., 2015), and furthermore it was demonstrated that the levels of IL-6 rise with disease progression as a part of systemic inflammatory response (Lu et al., 2016). IL-6 produced outside the CNS can cross the blood-brain barrier and might further exacerbate the response within the CNS (Garbuzova-Davis et al., 2018), causing further glial activation and cytotoxicity, eventually leading to motor neuron death (Milligan et al., 2021).

Another source of IL-6 –directly within the CNS – is microglia, which exist in a range of phenotypes between M1 and M2 (Guo et al., 2022). M1 phenotype releases IL-6 during chronic activation in neurodegenerative processes, while M2 microglia release anti-inflammatory mediators and exert neuroprotective effects (Muzio et al., 2021).

Imaging studies in ALS patients, ALS mouse models and human post-mortem tissue samples provide evidence that microglia act pro-inflammatorily (Calafatti et al., 2023). In familial ALS caused by mutations in SOD1, as well as in ALS-SOD1 mouse models, it was shown that microglia mediate death aof motor neurons through an NF-κB dependent mechanism and by producing pro-inflammatory cytokines (including IL-6), reactive oxygen species and tumour necrosis factor (Parisi et al., 2013). Another study using SOD1 mouse model of ALS demonstrated that the onset of the disease is associated with activation of microglia and production of IL-6, TNF-α, and IL-1β, indicating that the mutant SOD1 protein can induce a pathogenic response in microglia (Heneka et al., 2014).

Given the described role of microglial IL-6 in the pathogenesis of ALS, it should be investigated how its levels influence the progression of motor neuron degeneration, which might be then used to control the inflammatory response within the CNS and ultimately slow down the disease progression.

Hypothesis and aims

The hypothesis of this research is that the modulation of microglial IL-6 levels influences the progression of motor neuron degeneration in a mouse model of ALS.

The following aims are proposed:

1. Assessing of the expression levels of microglial IL-6 in a mouse model of ALS.
2. Investigation of the impact of microglial IL-6 modulation on neuroinflammation, survival of motor neurons and disease progression.
3. Evaluation of the therapeutic potential of targeting IL-6 signalling in microglia.

Experimental plan

Mouse model selection

In this research, the SOD1^{G93A} transgenic mice will be used. This well-characterized model was selected due to its documented relevance to neuroinflammation and microglia involvement (Geloso et al., 2017) and its recapitulation of most of the pathophysiology of ALS. However, it should be noted that this model does not replicate motor neuron degeneration in the cerebral cortex as seen in humans (Zhu et al., 2023), and additional research might be needed to investigate this topic specifically in the cortical region or in the context of the cortical involvement.

To obtain appropriate control groups, wild-type (WT) mice will be crossed with SOD1 ^{G93A} mice and the offspring will be genotyped to identify the transgenic ones (Calvo et al., 2012), ensuring the WT ones will be genetically closely matched.

Aim 1: Assessing of the expression levels of microglial IL-6 in a mouse model of ALS.

The purpose of this aim is to establish baseline levels of microglial IL-6 and their correlation with different disease stages.

To understand the role of microglial IL-6 at the different stages of the disease, tissue samples (spinal cord and brainstem) will be collected from SOD1^{G93A} mice and age-matched controls in three age groups: 8 weeks old, 12 weeks old, and 16 weeks days old, representing pre-symptomatic, early symptomatic and late symptomatic stages, respectively (González-Fernández et al., 2016).

For the isolation of microglia from the obtained CNS tissue sections, immuno-staining and fluorescence activated cell sorting (FACS) will be used. While this method is slower and slightly less efficient than magnetic activated cell sorting (MACS), the obtained microglia tend to be purer and less contaminated (Pan & Wan, 2020).

Next, RNA will be extracted from the microglia using the commercially available RNA extraction set, so that complementary DNA (cDNA) can be synthesized through reverse

transcription and then used as the template for the quantitative PCR (qPCR) reaction (Kuang et al., 2018).

To quantify IL-6 expression levels between WT and SOD ^{G93A} mice, qPCR will be employed to measure IL-6 mRNA, using primers that target the regions near the 5' and 3' ends of the coding sequence (Annibalini et al., 2012). The results will be compared using the relative quantification method, with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the reference gene, allowing to determine fold changes in IL-6 mRNA (Lim et al., 2020), followed by statistical analysis to evaluate the significance of the differences.

To evaluate and compare the actual presence of IL-6 in the obtained tissue samples from both groups, the protein levels will be measured using western blotting chemiluminescence reagents (Sukoff Rizzo et al., 2012), with normalization of the results relative to GAPDH protein expression. Spatial localization of IC-6 expression was not considered of key importance for this aim and therefore will not be investigated. However, it might provide insight into the focality of IL-6 dysregulation and could therefore be investigated in a future study.

Aim 2: Investigation of the impact of microglial IL-6 modulation on neuroinflammation, survival of motor neurons and disease progression.

The purpose of this aim is to investigate how upregulation and downregulation of microglial IL-6 affects ALS progression to determine if it is a viable therapeutic target.

The investigation will be conducted in three experimental groups of SOD ^{G93A} mice: control (no modulation of IL-6), IL-6 upregulated and IL-6 downregulated.

Baseline measurements will be performed 1 week before the modulation and will include the following characteristics: body weight, rotarod performance to assess motor coordination and grip strength to evaluate hindlimb muscle strength (Lim et al., 2020). As SOD1 ^{G93A} mice generally become symptomatic around the age of 10 weeks (Gómez-Gálvez et al., 2024), the baseline measurements will be performed at week 9.

For baseline assessment of motor neuron counts and neuroinflammatory markers, a separate, small subset of SOD1 ^{G93A} mice will be used to provide spinal cord and brain sections. Motor neurons will be identified using immunofluorescent labelling of choline acetyltransferase (ChAT) and quantified by imaging the sections by confocal microscopy and manual analysis of the images obtained (Rudnick et al., 2017). Neuroinflammation will be assessed by measurement of microglia and astrocyte activation (Shen et al., 2023), using ionized calcium-binding adaptor molecule 1 (Iba1; rabbit, 1:600) and glial fibrillary acidic protein (GFAP; mouse; 1:400), respectively. The secondary antibodies used will be donkey Alexa 568-conjugated anti-rabbit IgG (1:1000) and donkey Alexa 488-conjugated anti-mouse IgG (1:500). Fluorescence images will be acquired and processed with a confocal laser scanning microscope using a 63x oil objective (Shen et al., 2023).

For upregulation of microglial IL-6, a method described by Rosario et al. (2016) and further optimized (though in retinal microglia) by Maes et al. (2021) will be used. This method uses capsid-modified recombinant adeno-associated virus type 6 (rAAV6) vectors for microglial transduction, and the authors of the original study tested the ability of triple-mutant AAV6

carrying the gene for IL-6 to express and affect brain tissue, resulting in a significant increase of both IL-6 protein and mRNA levels. In this case, selective targeting of microglia was enabled by the incorporation of a microglia-specific CD68 promoter (Rosario et al., 2016).

The same method will be used for downregulation of microglial IL-6, using triple-mutant AAV6 carrying short hairpin RNA (shRNA) complementary to the mRNA of the IL-6 gene to silence its expression via RNA interference (Lambeth & Smith, 2012). The designed shRNA sequences will be validated first to confirm the silencing effect, which will done by performing real-time qPCR (Taxman et al., 2010) and observing a significant decrease in IL-6 mRNA.

Using rAAVs was chosen due to their particular suitability for in vivo genome editing owing to their low immunogenicity and amenability to tissue tropism modification methodologies based on engineered capsids (Chen & Gonçalves, 2016).

To verify the results of the microglial IL-6 modulation, a small subset of mice of each group will be euthanized two weeks after AAV6 injection and IL-6 mRNA and protein levels will be measured using qPCR and western blotting as described in Aim 1. Insufficient consistency in achieved results might be a potential problem of the chosen method, which has not yet been widely adopted for microglia transduction (Ball et al., 2024).

Post-modulation measurements of the selected characteristics will be performed on groups of animals as follows: body weight will be recorded biweekly, starting 1 week after the modulation (Lim et al., 2020), rotarod performance and grip strength will be measured 2, 4 and 8 weeks after the modulation. Tissue analysis for assessment of neuroinflammatory markers and motor neuron counts, as described above, will be performed on different cohorts of mice biweekly, up to and including week 8 (post-modulation). Investigators will be blinded to the group of IL-6 modulation to avoid bias.

During the whole experiment, disease onset, data quantifying rate of progression and lifespan of each animal will be recorded.

Data gathered will be analysed and ANOVA will be used to compare variables per time point across the three groups to determine if there are significant differences and how they evolve over time (Acevedo-Arozena et al., 2011).

There are two anticipated outcomes that would support the hypothesis of this proposal: 1) mice in the IL-6 upregulated group demonstrate an increase in neuroinflammatory markers, reduced motor neuron counts and faster disease progression; 2) mice in the IL-6 downregulated group demonstrate reduced neuroinflammation, more stable motor neuron counts and slower disease progression.

Aim 3: Evaluation of the therapeutic potential of targeting IL-6 signalling in microglia.

The purpose of this aim is to assess the therapeutic efficacy of modulating IL-6 signalling in microglia on neuroinflammation, motor neuron count, and disease progression in $SOD1^{G93A}$ mouse model of ALS.

Targeting microglial IL-6 represents a more precise and selective intervention than targeting IL-6 from other sources but at the same time requires drug delivery systems or agents that

are specific to microglia, which is especially important as targeting microglia can be confounded by off-target effects on other resident macrophages or those derived from blood monocytes (Miron & Priller, 2020). This is in addition to the general challenges of CNS therapeutics – the need to overcome the blood-brain barrier and to maintain stability of their active form before the target site of pathology is reached (Zhao et al., 2020).

The currently mostly used strategies or their combination was considered: nanoparticles (NPs) and cell-penetrating peptides (CPPs), characterized by various uptake mechanisms (Wu & Angelova, 2023). For this proposal, NPs were chosen because their different types can conveniently target microglia (Zhao et al., 2020) and there are recent papers describing efficacy of the use of NPs for microglial targeting and inhibiting neuroinflammation in Alzheimer's disease (Gebril et al., 2024). Biocompatible materials such as biodegradable polymeric NPs will be preferred due to a lower risk of triggering an immune response, and to further improve the targeting capabilities, targeting ligands or peptides specific for microglial receptors can be incorporated in the structure (Zhao et al., 2020). To achieve this, poly-lactic-co-glycolic acid (PLGA) will be used to encapsulate the therapeutics and anti-CX3CR1 antibody will be used as the microglia-targeting ligand. This combination has been already used to reduce microglial activation in rats (Noh et al., 2020).

To block the IL-6 signalling pathway, two approaches will be employed: targeting IL-6 itself and targeting its receptors (Speake et al., 2022). For this purpose, currently existing agents will be used for each approach – antibody against IL-6 (siltuximab) and IL-6 receptor antagonist (tocilizumab). The agents will be encapsulated within the PLGA nanoparticles using a water/oil/water double emulsion solvent technique, which has been proven to be a feasible approach for encapsulating monoclonal antibodies (Gdowski et al., 2015).

Given the current progress in the intranasal PLGA-based drug delivery (Alghareeb et al., 2024), this route of administration was considered first. However, it is a less established method with its own significant challenges (Formica et al., 2022), which might introduce additional complexity in this research, and therefore it was decided to administer the drug intravenously and potentially investigate the intranasal administration in a follow-up study.

For the experiment, 6 groups of $SOD1^{G93A}$ transgenic mice will be needed: 2 control groups treated with the vehicle only (early and late disease), 2 groups treated with tocilizumab (early and late disease) and 2 groups treated with siltuximab (early and late disease). The mice in the early and late stages (in all 3 groups) will start treatment at the age of 10 weeks and 18 weeks, respectively.

To determine the dosage and frequency, literature review has been conducted. For tocilizumab in mice, 6 mg/kg (Hudobenko et al., 2017) to 8mg/kg once a week (Wu et al., 2018) has been reported, and one study stated 10mg/kg twice a week for siltuximab (Song et al., 2014). However, it is important to note that neither agent is expected to cross the blood-brain barrier (Riegler et al., 2019) and therefore for this research, where the nanoparticle-based delivery and encapsulation improves the delivery to the CNS, a lower dose will be used: 3.5 mg/kg for tocilizumab and 4mg/kg for siltuximab, i.e. 50 % of the original doses. Additional studies to assess pharmatokinetics and validate the proposed dosage would be beneficial.

The endpoint was defined as inability of the mouse to right itself within 30 s when placed on its side, accompanied by the bodyweight loss reaching 25 % of the peak (Guan et al., 2023).

To assess the therapeutic efficacy, disease progression, neuroinflammatory markers and motor neuron counts will be evaluated at defined time intervals for each group, using procedures described in the respective sections above. For each animal, disease onset, progression rate and survival will be recorded. Investigators will be blinded to treatment groups to avoid bias.

The data obtained will be analysed and individual metrics compared between treated and control groups. ANOVA will be used to evaluate significance of any differences found between the groups.

References

1. Abramzon, Y.A. *et al.* (2020) 'The overlapping genetics of amyotrophic lateral sclerosis and frontotemporal dementia', *Frontiers in Neuroscience*, 14. doi:10.3389/fnins.2020.00042.
2. Acevedo-Arozena, A. *et al.* (2011) 'A comprehensive assessment of the *sod1g93a* low-copy transgenic mouse, which models human amyotrophic lateral sclerosis', *Disease Models & Mechanisms*, 4(5), pp. 686–700. doi:10.1242/dmm.007237.
3. Alghareeb, S. *et al.* (2024a) 'PLGA nanoparticles for nasal drug delivery', *Journal of Drug Delivery Science and Technology*, 95, p. 105564. doi:10.1016/j.jddst.2024.105564.
4. Annibalini, G. *et al.* (2012) 'The expression analysis of mouse interleukin-6 splice variants argued against their biological relevance', *BMB Reports*, 45(1), pp. 32–37. doi:10.5483/bmbrep.2012.45.1.32.
5. Ball, J.B. *et al.* (2024) 'Use of adeno-associated viruses for transgenic modulation of microglia structure and function: A review of technical considerations and Challenges', *Brain, Behavior, and Immunity*, 118, pp. 368–379. doi:10.1016/j.bbi.2024.03.005.
6. Calafatti, M. *et al.* (2023) 'Microglial crosstalk with astrocytes and immune cells in amyotrophic lateral sclerosis', *Frontiers in Immunology*, 14. doi:10.3389/fimmu.2023.1223096.
7. Calvo, A.C. *et al.* (2012) 'Genetic biomarkers for ALS disease in transgenic sod1g93a mice', *PLoS ONE*, 7(3). doi:10.1371/journal.pone.0032632.
8. Chen, X. and Gonçalves, M.A. (2016) 'Engineered viruses as genome editing devices', *Molecular Therapy*, 24(3), pp. 447–457. doi:10.1038/mt.2015.164.
9. Endo, F., Komine, O. and Yamanaka, K. (2016) 'Neuroinflammation in motor neuron disease', *Clinical and Experimental Neuroimmunology*, 7(2), pp. 126–138. doi:10.1111/cen3.12309.
10. Formica, M.L. *et al.* (2022) 'On a highway to the brain: A review on nose-to-brain drug delivery using nanoparticles', *Applied Materials Today*, 29, p. 101631. doi:10.1016/j.apmt.2022.101631.
11. Garbuzova-Davis, S. *et al.* (2018) 'Potential role of humoral IL-6 cytokine in mediating pro-inflammatory endothelial cell response in amyotrophic lateral sclerosis', *International Journal of Molecular Sciences*, 19(2), p. 423. doi:10.3390/ijms19020423.

12. Galimberti, D. *et al.* (2015) 'Inflammatory molecules in frontotemporal dementia: Cerebrospinal fluid signature of Progranulin mutation carriers', *Brain, Behavior, and Immunity*, 49, pp. 182–187. doi:10.1016/j.bbi.2015.05.006.
13. Gdowski, A. *et al.* (2015) 'Development of biodegradable nanocarriers loaded with a monoclonal antibody', *International Journal of Molecular Sciences*, 16(2), pp. 3990–3995. doi:10.3390/ijms16023990.
14. Gebril, H.M. *et al.* (2024) 'Nanotechnology for microglial targeting and inhibition of neuroinflammation underlying alzheimer's pathology', *Translational Neurodegeneration*, 13(1). doi:10.1186/s40035-023-00393-7.
15. Geloso, M.C. *et al.* (2017) 'The dual role of microglia in ALS: Mechanisms and therapeutic approaches', *Frontiers in Aging Neuroscience*, 9. doi:10.3389/fnagi.2017.00242.
16. Gómez-Gálvez, P. *et al.* (2024) *Computational analysis of Sod1-G93A mouse muscle biomarkers for comprehensive assessment of ALS progression* [Preprint]. doi:10.1101/2024.03.11.584407.
17. González-Fernández, C. *et al.* (2016) 'Wnt signaling alteration in the spinal cord of amyotrophic lateral sclerosis transgenic mice: Special focus on frizzled-5 cellular expression pattern', *PLOS ONE*, 11(5). doi:10.1371/journal.pone.0155867.
18. Guo, S., Wang, H. and Yin, Y. (2022) 'Microglia polarization from M1 to M2 in Neurodegenerative Diseases', *Frontiers in Aging Neuroscience*, 14. doi:10.3389/fnagi.2022.815347.
19. Guan, T. *et al.* (2023) 'Selective removal of misfolded SOD1 delays disease onset in a mouse model of amyotrophic lateral sclerosis', *Cellular and Molecular Life Sciences*, 80(10). doi:10.1007/s00018-023-04956-9.
20. Heneka, M.T., Kummer, M.P. and Latz, E. (2014) 'Innate immune activation in neurodegenerative disease', *Nature Reviews Immunology*, 14(7), pp. 463–477. doi:10.1038/nri3705.
21. Hudobenko, J., Verma, R. and McCullough, L. (2017) 'Abstract TP270: Interleukin-6 receptor inhibition with tocilizumab ameliorates ischemic stroke damage in mice', *Stroke*, 48(suppl_1). doi:10.1161/str.48.suppl_1.tp270.
22. Kjældgaard, A.-L. *et al.* (2021) 'Prediction of survival in amyotrophic lateral sclerosis: A nationwide, Danish cohort study', *BMC Neurology*, 21(1). doi:10.1186/s12883-021-02187-8.
23. Kuang, J. *et al.* (2018) 'An overview of technical considerations when using quantitative real-time PCR analysis of gene expression in human exercise research', *PLOS ONE*, 13(5). doi:10.1371/journal.pone.0196438.
24. Lambeth, L. and Smith, C. (2012) 'Short hairpin RNA-mediated gene silencing', *Methods in Molecular Biology*, pp. 205–232. doi:10.1007/978-1-62703-119-6_12.
25. Liu, J. and Wang, F. (2017) 'Role of neuroinflammation in amyotrophic lateral sclerosis: Cellular mechanisms and therapeutic implications', *Frontiers in Immunology*, 8. doi:10.3389/fimmu.2017.01005.
26. Lim, C.K.W. *et al.* (2020) 'Treatment of a mouse model of ALS by in vivo base editing', *Molecular Therapy*, 28(4), pp. 1177–1189. doi:10.1016/j.ymthe.2020.01.005.
27. Lu, C.-H. *et al.* (2016) 'Systemic inflammatory response and neuromuscular involvement in amyotrophic lateral sclerosis', *Neurology Neuroimmunology & Neuroinflammation*, 3(4). doi:10.1212/nxi.0000000000000244.

28. Maes, M.E. *et al.* (2021) 'Optimizing AAV2/6 microglial targeting identified enhanced efficiency in the photoreceptor degenerative environment', *Molecular Therapy - Methods & Clinical Development*, 23, pp. 210–224. doi:10.1016/j.omtm.2021.09.006.
29. Milligan, C. *et al.* (2021) 'Tocilizumab is safe and tolerable and reduces c-reactive protein concentrations in the plasma and cerebrospinal fluid of als patients', *Muscle & Nerve*, 64(3), pp. 309–320. doi:10.1002/mus.27339.
30. Miron, V.E. and Priller, J. (2020) 'Investigating microglia in health and disease: Challenges and opportunities', *Trends in Immunology*, 41(9), pp. 785–793. doi:10.1016/j.it.2020.07.002.
31. Muzio, L., Viotti, A. and Martino, G. (2021) 'Microglia in neuroinflammation and neurodegeneration: From understanding to therapy', *Frontiers in Neuroscience*, 15. doi:10.3389/fnins.2021.742065.
32. Nichols, N.L. *et al.* (2013) 'Ventilatory control in ALS', *Respiratory Physiology & Neurobiology*, 189(2), pp. 429–437. doi:10.1016/j.resp.2013.05.016.
33. Noh, C. *et al.* (2020) 'CX3CR1-targeted plga nanoparticles reduce microglia activation and pain behavior in rats with spinal nerve ligation', *International Journal of Molecular Sciences*, 21(10), p. 3469. doi:10.3390/ijms21103469.
34. Pan, J. and Wan, J. (2020) 'Methodological comparison of FACS and Macs isolation of enriched microglia and astrocytes from Mouse Brain', *Journal of Immunological Methods*, 486, p. 112834. doi:10.1016/j.jim.2020.112834.
35. Parisi, C. *et al.* (2013) 'Dysregulated microRNAs in amyotrophic lateral sclerosis microglia modulate genes linked to neuroinflammation', *Cell Death & Disease*, 4(12). doi:10.1038/cddis.2013.491.
36. Riegler, L.L., Jones, G.P. and Lee, D.W. (2019) ' current approaches in the grading and management of cytokine release syndrome after chimeric antigen receptor T-cell therapy ', *Therapeutics and Clinical Risk Management*, Volume 15, pp. 323–335. doi:10.2147/tcrm.s150524.
37. Rosario, A.M. *et al.* (2016) 'Microglia-specific targeting by novel capsid-modified Aav6 vectors', *Molecular Therapy - Methods & Clinical Development*, 3, p. 16026. doi:10.1038/mtm.2016.26.
38. Rudnick, N.D. *et al.* (2017) 'Distinct roles for motor neuron autophagy early and late in the sod1 g93a mouse model of Als', *Proceedings of the National Academy of Sciences*, 114(39). doi:10.1073/pnas.1704294114.
39. Scheller, J. *et al.* (2011a) 'The pro- and anti-inflammatory properties of the cytokine interleukin-6', *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, 1813(5), pp. 878–888. doi:10.1016/j.bbamcr.2011.01.034.
40. Shen, Y. *et al.* (2023) 'Ultrasound–enhanced brain delivery of Edaravone provides additive amelioration on disease progression in an ALS mouse model', *Brain Stimulation*, 16(2), pp. 628–641. doi:10.1016/j.brs.2023.03.006.
41. Song, L. *et al.* (2014) 'Antitumor efficacy of the anti-interleukin-6 (IL-6) antibody siltuximab in mouse xenograft models of lung cancer', *Journal of Thoracic Oncology*, 9(7), pp. 974–982. doi:10.1097/jto.0000000000000193.

42. Speake, C. *et al.* (2022) 'IL-6–targeted therapies to block the cytokine or its receptor drive distinct alterations in T cell function', *JCI Insight*, 7(22). doi:10.1172/jci.insight.159436.

43. Sukoff Rizzo, S.J. *et al.* (2012) 'Evidence for sustained elevation of IL-6 in the CNS as a key contributor of depressive-like phenotypes', *Translational Psychiatry*, 2(12). doi:10.1038/tp.2012.120.
44. Talbott, E.O., Malek, A.M. and Lacomis, D. (2016) 'The epidemiology of amyotrophic lateral sclerosis', *Neuroepidemiology*, pp. 225–238. doi:10.1016/b978-0-12-802973-2.00013-6.
45. Taxman, D.J. *et al.* (2010) 'Short hairpin RNA (shrna): Design, delivery, and assessment of Gene Knockdown', *Methods in Molecular Biology*, pp. 139–156. doi:10.1007/978-1-60761-657-3_10.
46. Tortelli, R. *et al.* (2020b) 'Plasma inflammatory cytokines are elevated in ALS', *Frontiers in Neurology*, 11. doi:10.3389/fneur.2020.552295.
47. Wu, R. *et al.* (2018) 'IL-6 receptor blockade ameliorates diabetic nephropathy via inhibiting inflammasome in mice', *Metabolism*, 83, pp. 18–24. doi:10.1016/j.metabol.2018.01.002.
48. Wu, Y. and Angelova, A. (2023) 'Recent uses of lipid nanoparticles, cell-penetrating and bioactive peptides for the development of brain-targeted nanomedicines against neurodegenerative disorders', *Nanomaterials*, 13(23), p. 3004. doi:10.3390/nano13233004.
49. Zhao, N. *et al.* (2020) 'Microglia-targeting nanotherapeutics for neurodegenerative diseases', *APL Bioengineering*, 4(3). doi:10.1063/5.0013178.
50. Zhu, L. *et al.* (2023) 'Pathological insights from amyotrophic lateral sclerosis animal models: Comparisons, Limitations, and challenges', *Translational Neurodegeneration*, 12(1). doi:10.1186/s40035-023-00377-7.