nature
REVIEWS

Nature Reviews referee guidelines

Review articles

Nature Reviews publishes timely, authoritative articles that are of broad interest and exceptional quality. Thank you for taking the time to help us to ensure that our articles meet these high standards.

Review articles in *Nature Reviews* journals provide accessible, authoritative and balanced overviews of a field or topic. These articles are targeted towards readers from advanced undergraduate level and upwards, including researchers, academics and clinicians, and should be accessible to readers working in any discipline.

Please submit your report in narrative form and provide detailed justifications for all statements. Confidential comments to the editor are welcome, but it is helpful if the main points are stated in the comments for transmission to the authors.

Please note that all *Nature Reviews* articles will be thoroughly edited before publication and all figures will be redrawn by our in-house art editors. We therefore request that you concentrate on the scientific content of the article, rather than any minor errors in language or grammar.

Please consider and comment on the following points when reviewing this manuscript:

- Is the article timely and does it provide a useful addition to the existing literature?
- Are the scope and aims of the article clear?
- Are the ideas logically presented and discussed?
- Is the article accessible to a wide audience, including readers who are not specialists in your own field?
- Does the article provide a balanced overview of the literature? Please bear in mind that it may not be possible to cover all aspects of a field within such a concise article.
- Does the article provide new insight into recent advances?
- Is the discussion fair and accurate? Although our authors are encouraged to be opinionated, they should not ignore alternative points of view.
- Do the figures, boxes and tables provide clear and accurate information? Are there any additional or alternative display items that you think that the authors should include?
- Are the references appropriate and up-to-date? Do they reflect the scope of the article?
- Are you aware of any undeclared conflicts of interest that might affect the balance, or perceived balance, of the article?

TITLE PAGE

Title:

Non-neuronal cells in amyotrophic lateral sclerosis – pathogenesis to biomarkers

Authors:

Björn F. Vahsen[a], Elizabeth Gray[a], Alexander G. Thompson[a], Olaf Ansorge[a], Daniel C. Anthony[b], Sally A. Cowley[c], Kevin Talbot[a], Martin R. Turner[a]

Affiliations:

[a] Oxford Motor Neuron Disease Centre, Nuffield Department of Clinical Neurosciences, University of Oxford, John Radcliffe Hospital, Oxford OX3 9DU, United Kingdom

[b] Department of Pharmacology, University of Oxford, Mansfield Road, Oxford OX1 3QT, United Kingdom

[c] James Martin Stem Cell Facility, Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, United Kingdom

Corresponding authors:

Martin Turner, Email: martin.turner@ndcn.ox.ac.uk

Kevin Talbot, Email: kevin.talbot@ndcn.ox.ac.uk

Abstract

A motor neuron centric view of pathogenesis may be a significant factor in the failure to identify disease-modifying therapy for the neurodegenerative disorder amyotrophic lateral sclerosis (ALS). Non-neuronal cells have crucial homeostatic functions within the central nervous system, and evidence of involvement in the pathophysiology of several neurodegenerative disorders, including ALS, is accumulating. Microglia and astrocytes, in crosstalk with peripheral immune cells, may exert both neuroprotective and adverse effects, indicating the highly nuanced nature of neuronal and non-neuronal cell interactions. This review provides an overview of the physiological roles of non-neuronal cells in relation to the pathogenesis of ALS, and the emerging potential of non-neuronal cell biomarkers to advance therapeutic development.

Introduction

Amyotrophic lateral sclerosis (ALS) is the third commonest neurodegenerative disorder after Alzheimer's disease (AD) and Parkinson's disease (PD), and is characterised by the loss of cortical, brainstem and spinal anterior horn motor neurons. Progressive paralysis typically results in death from neuromuscular respiratory failure, with a median survival from symptom onset of 30 months. However, there is significant clinical heterogeneity in site of onset, pattern of progression, variation in rate of progression and overall survival. Together with the clinicopathological overlap with frontotemporal dementia (FTD), this indicates that the pathophysiology is driven and modified by complex biological factors[1].

The aetiology of most ALS cases is still unclear, but a mutation in a single gene is found in up to 15%, including familial and apparently sporadic cases, with multiple genes involved[2]. The commonest is a dynamic hexanucleotide repeat expansion in the first intron of *C9orf72*, found in ~40% of familial ALS (and FTD) cases[3,4], while ~20% are associated with mutations in *SOD1*[5], and less than 5% each with mutations in the *TARDBP* (encoding TDP-43)[6] and *FUS* genes[7]. Nearly all cases of ALS (and 50% of FTD) are associated with characteristic nuclear clearing and cytoplasmic aggregation of the 43kDa transactive response DNA binding protein TDP-43[8]. This neuropathological signature occurs in both neuronal and non-neuronal cells, with the exception of the 3% of all ALS cases associated with mutations in the *SOD1* and *FUS* genes. The relative selectivity and non-random pattern of degeneration of cortical and spinal cord motor neurons suggests that ALS is a system degeneration, but genetic heterogeneity also points to a clinicopathological syndrome arising from a broad range of upstream converging biological processes[1].

In experimental models, multiple motor neuron-intrinsic, so-called cell-autonomous, pathways have been implicated in ALS pathogenesis, including mitochondrial dysfunction, axonal

transport, RNA metabolism and nucleocytoplasmic transport[9]. Although most show some response to therapeutic modulation in disease models, clinical translation has proved elusive. Riluzole and the free-radical scavenger edaravone are the only licensed disease-modifying drugs for ALS, with extremely limited impact on survival and rate of progression respectively.

One of the potential reasons for the recurrent failure of clinical trials is the focus of pre-clinical models on motor neurons. Non-neuronal cells exert crucial homeostatic functions in the central nervous system (CNS), and an increasing body of evidence now implicates their involvement in ALS pathogenesis. This pathogenic involvement of non-neuronal cells in neurological disease is commonly referred to as "neuroinflammation". However, the meaning of this term is context-dependent and not clearly defined in relation to the specific cell types to which it refers. In rodent models of ALS, non-neuronal cells exert both harmful and protective, so-called non-cell-autonomous, influences on neurons. To overcome the limitations of these models imposed by species-specific differences in cellular function, induced pluripotent stem cell (iPSC)-derived cells have the potential to provide new insights into the specific functions of human non-neuronal cells pertinent to neurodegenerative disorders, including ALS. A further major barrier to therapeutic development in neurodegenerative disorders such as ALS is the lack of objective human biofluid markers of disease activity, and molecules derived from non-neuronal cells are of increasing interest as potential biomarkers[10].

In this review we outline the role of non-neuronal cells in CNS physiology, with particular focus on microglia, astrocytes, and oligodendrocytes. We consider the evidence for their involvement in ALS pathogenesis, and the prospects for novel biomarkers against which to assess emerging therapeutics.

Non-neuronal cells in the CNS

Non-neuronal cells in the CNS comprise those resident in the parenchyma, most importantly glia (subdivided into microglia, astrocytes, oligodendrocytes and oligodendrocyte progenitors NG2-glia) but also pericytes, endothelial, and ependymal cells. The prevailing dogma that glial cells significantly outnumber neurons has been questioned recently, with a glia-neuron ratio suggested to be nearer 1:1[11]. The perception of glia as passive 'bystanders' has been overcome by research showing that all glial cell types are of high functional importance in CNS development and physiology[12,13]. In addition, CNS-associated macrophages and peripheral non-neuronal cells, such as blood monocytes and macrophages, granulocytes and lymphocytes, and natural killer (NK) cells, reside on the borders of the CNS[14]. There is emerging evidence that these non-resident non-neuronal cells can also exert relevant physiological functions in the CNS[14].

In the following sections, we introduce the different non-neuronal cell types in the CNS, including their physiological functions and the evidence from human *post mortem* and neuroimaging studies for their involvement in ALS.

Glia in CNS physiology and human ALS

Microglia

Microglia (~5-10% of glial cells[11,15]) are the resident macrophages of the CNS, originating from yolk sac-derived precursors that migrate into the CNS during neurodevelopment[16,17] and maintain their population by self-renewal[18]. Depending on the surrounding milieu, microglia are either in a resting or activated state[19]. Previously considered dormant under homeostatic conditions, microglia are in fact highly active cells in their resting state, continually surveying and scanning the environment with their highly motile ramified processes[20]. Detection of any

disturbance to CNS homeostasis leads to microglial activation, resulting in rapid changes in their morphology, associated with alterations in their gene expression and functional behaviour[21,22]. Microglial activation has been classically conceptualized as one of two polarized stages: a pro-inflammatory M1 and an anti-inflammatory M2 phenotype[23-25] (for an overview on respective markers and cytokines, see a recent review[26]). Broadly, M1 microglia are thought to induce or exacerbate neuron dysfunction, while M2 microglia are considered neuroprotective[27,28]. However, it is now recognised that this is an overly simplistic view of microglia physiology and that their state of activation should rather be seen as a spectrum that is dependent on age, location, and the nature of the environmental challenge[21-23]. For example, constitutive MHC class II expression on microglia is higher in cerebellar white matter and the lumbar spinal cord than elsewhere in the brain[29]. Using this variety of activation states and their immense capacity to interact with their environment, microglia execute crucial functions in the maintenance of CNS homeostasis by providing nurture and support to neurons and other cells via the secretion of soluble factors, synaptic refinement, clearance of dead cells or mis-folded proteins, and protection from infectious agents[30].

In human ALS[31,32], and in pre-symptomatic carriers of *SOD1* mutations[33], widespread cerebral microglial activation was demonstrated by PET imaging *in vivo*. In *post mortem* ALS, elevated levels of microglial transcripts, increased microglial density, and activated microglial morphology have been found in addition to motor neuron degeneration[34,35]. Microglial activation correlated with neuronal and axonal loss, and was present in individuals that exhibited more rapid disease progression and greater burden of clinical upper motor neuron signs, but it has been hard to establish whether this association is causal or a consequence of the accelerated pathology[35,36]. Carriers of the *C9orf72* expansion showed particularly extensive microglial activation *post mortem*[26,35].

Astrocytes

Astrocytes (~20-40% of glial cells[11,37]), which originate from the neuroepithelium and share a common progenitor with neurons and oligodendroglia, control homeostasis in the CNS[37,38]. In close cross-talk with microglia, astrocytes maintain ion, neurotransmitter and energy homeostasis, perform synapse pruning, and provide trophic support to neurons[37,39]. Disturbance to CNS homeostasis leads to the transformation to reactive astrocytes, accompanied with substantial transcriptomic changes[40,41]. Corresponding to the M1/M2 concept used widely to categorize microglia phenotypes, A1 and A2 phenotypes have been recently proposed to broadly classify the diverging reaction of astrocytes to different stimuli[39,42]. Similar to their microglial counterparts, A1 astrocytes are proposed to have neurotoxic properties, while the A2 phenotype is connected to astrocyte-mediated neuroprotection[42-45].

In ALS *post mortem* tissue, similar to microglia, an increased presence of reactive astrocytes has been described, in addition to degenerating motor neurons[46-50], corroborated indirectly *in vivo* using PET to demonstrate cerebral white matter and pontine astrocytosis in patients[51].

Oligodendrocytes and NG2-glia

Oligodendrocytes (~50-75% of glial cells[11,52]) are myelin-producing cells in the CNS originating from precursors cells called NG2-glia[53,54]. In addition to their canonical function in neuronal action potential propagation, key roles of oligodendrocytes in providing metabolic support to neurons have been described[54]. In concert with astrocytes, oligodendrocytes maintain axonal integrity, survival, and adaptation[54,55]. Interestingly, NG2-glia have recently been implicated in modulating microglial function[56].

Oligodendrocyte pathology has been found in multiple areas of the CNS in ALS[46,47,57], and its relevance underscored by the consistent presence of TDP-43 inclusions in oligodendrocytes[57].

Non-glial non-neuronal cells

CNS-associated macrophages

In addition to microglia, a distinct population of CNS-associated meningeal, perivascular, and choroid-plexus macrophages are recognised[58,59]. These cells form a stable population residing physiologically at the CNS interface and do not enter the CNS parenchyma[14,59]. Their detailed physiological function is only beginning to emerge; currently they are considered important for controlling the permeability of the blood-brain barrier (BBB), and the transport of metabolites to and from the CNS[58].

Peripheral immune cells

The entry of peripheral immune cells into the CNS parenchyma is highly restricted in a healthy state, and only activated $CD4^+$ T-cells, and not $CD8^+$ T-cells, traffic into the CNS regardless of the antigen specificity[60], while neutrophils and B-cells are rarely, if ever, found in the healthy brain parenchyma[14,61]. It remains unclear why only specific leukocyte populations infiltrate, but there is low basal expression of MHC class I on endothelial cells and there is no basal expression of MHC class II. Upon injury, however, multiple peripheral immune cell types, both innate and adaptive, such as granulocytes, monocyte-derived macrophages, lymphocytes, and NK cells, can be recruited from the blood circulation across the BBB[14,61]. There is emerging evidence for close cross-talk and communication between these infiltrating cells and resident glial cells, modulating glial function in both protective and harmful ways[14].

In ALS *post mortem* tissue, peripheral cells, such as $CD4^+$ and $CD8^+$ T-cells, macrophages, and NK cells, infiltrate multiple areas of the CNS[34,46,62], suggesting that there is a contribution of immune-mediated events to the pathogenesis of ALS.

Key questions based on human pathology

These findings outline a key role for CNS and peripheral non-neuronal cells in ALS, raising the questions of whether the observed involvement of non-neuronal cells is beneficial or detrimental, if different cell types play divergent roles in the pathophysiology and whether their roles change over the ALS disease course, and if non-neuronal cell involvement is a primary driver of neurodegeneration or a secondary reaction to tissue injury.

Non-neuronal cells in ALS models

Aiming to answer the key questions arisen from human neuroimaging and *post mortem* studies, researchers have employed a variety of experimental ALS models (**Table 1)**. The following sections examine how *in vitro and in vivo* models for the commonest mutations in *SOD1*, *TDP-43*, and *C9orf72* have improved understanding of the functional role of different non-neuronal cell types in ALS by providing mechanistic insight into their contribution to ALS pathophysiology and pathogenesis including disease onset and progression.

Microglia in ALS

SOD1 models

Transgenic animals expressing a mutant form of human SOD1 (mSOD1) recapitulate some of the key clinical features of human ALS such as progressive motor abnormalities and paralysis, with pathological evidence of protein aggregation, motor neuron degeneration and gliosis, and have thus been widely used as a disease model[63-65]. Corresponding to the results obtained from human *post mortem* tissue, microglial activation is reported consistently in SOD1 models and, in some studies, occurs well before the onset of clinical disease[66-68]. Selective expression of different forms of mSOD1 in motor neurons was found to be insufficient to induce motor neuron degeneration[69,70] or, with higher transgene expression, resulted in mild phenotypes[71,72].

Although it is difficult to compare these results with the phenotypes found in animals ubiquitously expressing mSOD1, these data underscore the importance of non-cell-autonomous factors from non-neuronal cells in the disease pathogenesis. The expression of $SOD1^{G37R}$ in motor neurons was found to determine disease onset and early disease progression, while selective expression of $SOD1^{G93A}$ in microglia was not sufficient to cause motor neuron degeneration[73]. Similarly, shRNA-mediated silencing of $SOD1^{G93A}$ in motor neurons delayed the disease onset but had no effect on the progression once the disease commenced[74], whereas deletion of $SOD1^{G37R}$ in microglia substantially slowed later disease progression, but did not significantly alter earlier phases of disease[75].

These data indicate that the observed activation of microglia, in SOD1 models at least, seems to be a secondary mechanism in response to neuronal damage and leading to disease exacerbation rather than a primary disease driver. This notion was further corroborated by an assessment of pro- and anti-inflammatory markers at early and late disease stages. Microglia isolated from $SOD1^{G93A}$ mice at disease onset showed higher expression of M2- and lower levels of M1-associated markers, respectively, compared with end stage disease[76]. Furthermore, $SOD1^{G93A}$ M2 microglia isolated at disease onset were neuroprotective in co-culture with motor neurons, whereas $SOD1^{G93A}$ M1 microglia from end-stage disease were neurotoxic[76]. Mechanistically, the anti-inflammatory cytokine IL-10 was up-regulated at pre-symptomatic stages, with disease onset delayed by overexpression of IL-10 and precipitated by antibody-mediated blockade[77]. However, several studies demonstrated that mSOD1-expressing microglia increase the production of neurotoxic factors. Compared with wild-type microglia, cultured primary $SOD1^{G93A}$ microglia released more reactive oxygen species (ROS), such as superoxide, and nitric oxide (NO) and less insulin-like growth factor (IGF-1), reducing the survival of co-cultured primary neurons, both in naïve and LPS-stimulated conditions[73,78] (FIG. 1). As a proof of principle, pre-treatment with L-NIL, an iNOS inhibitor, increased motor

neuron survival in co-culture[78]. In line with these results, treatment of mSOD1^{G93A} microglia with mSOD1^{G93A} recombinant protein, in comparison with SOD1^{WT} treatment, led to a strong increase in TNF-α, IL-1β, and superoxide release as well as iNOS expression, while IGF-1 production declined, and caused motor neuron toxicity in co-culture[79]. Again, this neurotoxicity was rescued by concomitant application of L-NIL in combination with the NADPH oxidase inhibitor apocynin[79]. While increased TNF-α release was also found after LPS-stimulation of cultured primary SOD1^{G93A} microglia compared with wild-type microglia[80], TNF-α KO in SOD1^{G93A} and SOD1^{G37R} mice did not affect life span or motor neuron loss[81], demonstrating that TNF-α is likely not a direct mediator of neurotoxicity in SOD1-ALS. In contrast, IL-1β KO slowed disease progression in SOD1^{G93A} mice[82] but did not alter the disease course in SOD1^{G37R} mice[83], suggesting that its neurotoxic effect is model-dependent.

Overall, the evidence currently points to early neuroprotective effects followed by a transition to toxic properties for microglia over the disease course in SOD1 models, during which both loss of neurotrophic support and gain-of-function toxicity play a role. However, it appears likely that this is an oversimplification of the true microglial disease phenotype. While many studies focused on specific M1/M2-associated markers, another study demonstrated by RNA sequencing that microglia isolated from SOD1^{G93A} mice showed concurrent expression of both neuroprotective and toxic factors at different time points during disease progression, thereby differing from M1/M2 phenotypes and suggesting the existence of an ALS-specific microglial phenotype that overlaps with other neurodegenerative diseases[84]. Future studies analyzing microglial gene and protein expression using unbiased "omics" approaches in pre-symptomatic, early and late stages of the disease are necessary to shed light on the detailed functional role of microglia in SOD1 models.

TDP-43 models

The discovery of cytoplasmic aggregates of TDP-43 as the signature common to 97% of all ALS cases was followed by their striking absence in those associated with *SOD1* mutations. Although the human clinical syndromes of *SOD1* and non-*SOD1* mutation-associated ALS have many features in common, these molecular developments have marked a significant shift away from the use of *SOD1* transgenic models.

In TDP-43 models, cellular dysfunction is currently thought to arise from a combination of cytoplasmic mislocalization-induced toxic gain-of-function and loss of nuclear TDP-43. Most studies have focused on the cell-autonomous effects of neuronal expression of mutant TDP-43 or TDP-43 aggregates, but some studies have also analyzed the microglial response to mutant TDP-43 expression or exogenous treatment with TDP-43. In both human ALS *post mortem* cortex tissue and mice expressing TDP-43^{A315T}, which developed time-dependent gait abnormalities, motor neuron degeneration, and premature death[85], microgliosis and rod-shaped microglia (i.e. a reactive microglial phenotype described in many encephalopathies, such as neurosyphilis) have been found in addition to degenerating upper motor neurons[86].

However, this does not resolve the issue as to whether microglia are actively involved in the disease pathogenesis in TDP-43 models or merely show a response, harmful or protective, to neuronal damage. Cell culture experiments have aimed to study their functional role. The addition of wild-type and different forms of mutant TDP-43 to primary microglial cultures leads to microglial activation and upregulation of pro-inflammatory markers, such as TNF-α and IL-1β (FIG. 1), with mutant TDP-43 eliciting a more pronounced response[87,88]. While treatment of motor neurons with TDP-43^{A315T} caused no toxicity in the absence of microglia, neurotoxic

effects were observed in co-culture[87]. TDP-43 aggregates added to primary microglia cultures were internalized, also leading to the release of pro-inflammatory factors including IL-1β[89].

While these results are in line with the conceived neurotoxic microglial response in SOD1 models of ALS, there is conflicting evidence from other TDP-43 models. In a mouse model in which neuronal cytoplasmic TDP-43 aggregation could be reversibly induced in by expression of TDP-43 lacking the nuclear localization sequence, progressive motor dysfunction and motor neuron loss was associated with only subtle microglial changes[90]. However, the subsequent suppression of neuronal mutant TDP-43 expression caused a drastic proliferation of microglia, leading to the clearance of neuronal TDP-43 aggregates and functional recovery. It is unclear why this only occurs once the expression of the transgene is halted, unless mutant TDP-43 expression actually inhibited microglial activity directly, or downregulation of endogenous and nuclear TDP-43 reduced the normal expression of factors driving neuronal-microglial crosstalk. The application of PLX3397, a CSF1R/c-kit inhibitor that depletes microglia, resulted in impaired motor function of these mice, demonstrating a neuroprotective effect of microglia in this model[90]. While these results were surprising, the depletion of microglia eliminates the parallel neurotoxic and neuroprotective contribution of this population and thus it is hard to evaluate the relative contribution of microglia to death or survival in this model. Supporting the neuroprotective contribution of microglia, in zebrafish expressing TDP-43 in motor neurons UV-induced neuronal injury led to neurodegeneration and TDP-43 redistribution and release, which was prevented by microglial phagocytosis[91]. It is noteworthy that in both studies wild-type microglia were used. Therefore, it is uncertain whether microglia expressing mutant TDP-43 or TDP-43 aggregates can exert similar neuroprotective effects. Aggregated TDP-43 was found in microglia in *post mortem* tissue and TDP-43 was experimentally shown to be a regulator of microglial phagocytosis via modulation of lysosomal biogenesis[57,92]. Conditional knock-out of TDP-43 in microglia promoted the phagocytic clearance of β-amyloid in a mouse

model of AD, but it also led to enhanced synapse loss[92]. Importantly, less β-amyloid and increased microglial activation were found in ALS *post mortem* tissue with TDP-43 pathology[86,92]. Mutant TDP-43 or TDP-43 aggregates in human microglia could therefore skew microglial properties towards neurotoxicity through gain-of-function toxicity due to cytoplasmic mislocalization or loss-of-function toxicity due to loss of nuclear TDP-43. Whether these beneficial microglial effects are translatable into human disease therefore remains unclear and requires validation in models with combined neuronal and microglial TDP-43 pathology.

C9orf72 models

Microglia have the highest *C9orf72* expression of any cell type in the brain, and analysis of *post mortem* tissue from ALS patients carrying the *C9orf72* expansion has confirmed microglial activation and upregulation of inflammatory pathways[93]. Three hypotheses for the pathophysiological effect of the hexanucleotide expansion in *C9orf72* have been made: loss-of-function through reduced *C9orf72* expression (haploinsufficiency), gain-of-function toxicity due to the formation of RNA foci, or primarily through repeat associated non-ATG (RAN) translated dipeptide repeat proteins (DPRs)[94]. Hence, the role of microglia has been examined in the context of loss- versus gain-of-function toxicity.

Most *C9orf72* transgenic mouse lines show widespread production of DPRs and RNA foci, and therefore demonstrate key gain-of-function mechanisms of the *C9orf72* mutation, but with no overt ALS motor phenotype, motor neuron degeneration, or inflammatory response[95-97]. Other studies have focused on analyzing the effects of the selective expression of specific DPRs. Poly(GA) inclusions are the most abundant in patients[98], and mouse models with neuron-specific expression of poly(GA) demonstrate motor deficits and microglial activation[99-101]. Neuronal poly(GA) expression was found to be associated with immune and cytokine pathway activation[99,101], in which microglia isolated from end-stage poly(GA) mice showed a pro-

inflammatory phenotype with pronounced activation of interferon-associated genes[101]. Presymptomatic treatment of poly(GA) mice with an anti-GA antibody has been reported to reduce poly(GA) inclusions, motor deficits, the levels of neurofilament light chains as a proxy for axonal damage, cytoplasmic TDP-43 mislocalization, and microglial activation[99]. The authors hypothesized that anti-GA antibody treatment might neutralize secreted poly(GA) or enhance its degradation, and attenuate a harmful microglial response[99].

Mice expressing poly(PR), the most neurotoxic DPR based on *in vitro* analyses, showed no clear ALS phenotype or microglial activation in one study[101] but with motor neuron loss and microglial activation in another[102]. Poly(GR) mice show neuronal cell loss and microgliosis[103,104]. Mouse models expressing other DPRs are yet to be published, but there seems to be consensus that neuronal DPR inclusions induce microglial activation. Whether this leads to neuroprotection or enhanced toxicity remains unresolved.

The consequences of the *C9orf72* expansion induced loss-of-function on microglia have also been investigated. *C9orf72*$^{+/-}$ and *C9orf72*$^{-/-}$ mice failed to develop clinical ALS or show evidence of neurodegeneration[93,96,105]. Instead, progressive splenomegaly, lymphadenopathy, and upregulation of pro-inflammatory cytokines in the periphery were found in *C9orf72*$^{-/-}$ mice, outlining a crucial role of *C9orf72* in the immune system[93,96,106]. Reduced expression of *C9orf72* led to the upregulation of microglial activation genes, increased levels of the pro-inflammatory cytokines IL-1β and IL-6, and caused accumulation of lysosomal markers[93,107].

Together, increasing neuronal dysfunction and aggregate release from diseased *C9orf72* neurons in concert with concomitant *C9orf72* loss-of-function in microglia might skew microglia to a neurotoxic phenotype. This notion is underscored by a study in which the strongest neurotoxicity was observed after concomitant hexanucleotide repeat expression and

C9orf72 knock-out[108] which in combination also induced the strongest microglial response. However, most results were significantly more pronounced in homozygous *C9orf72* knock-out mice, which do not recapitulate the rather mild reduction in *C9orf72* levels observed in *C9orf72* expansion carriers. Similarly, although *C9orf72* mutation-dependent RNA foci formation have been described in microglia[107], their potential impact on microglial function has not been evaluated to date. DPR inclusions have thus far not been demonstrated in microglia in human *post mortem* tissue or *C9orf72*-based animal models[109]. There is a need for more authentic disease models, in which gain-of-function and loss-of-function toxicity can be concomitantly modeled in both neurons and microglia. Only then, it will be possible to disentangle the effects of *C9orf72* mutations on microglia and their pathophysiological role in crosstalk with diseased neurons.

Summary

Taken together, clear microglial activation is found in SOD1, TDP43, and C9orf72 models of ALS. While neuroprotective microglial properties have been found in some paradigms, mostly in early disease phases in SOD1 animals, there is currently a relative consensus across these different models that the expression of mutations in these genes in microglia, together with primary motor neuron damage and release of protein aggregates, results in a pro-inflammatory microglial phenotype. Evidence from SOD1 models demonstrates that this pro-inflammatory phenotype causes neurotoxicity via soluble mechanisms. For TDP-43 and C9orf72 models, however, a direct neurotoxic effect of pro-inflammatory microglia has not yet been conclusively demonstrated. This is warranted in order to refine our understanding of whether microglial activation can be targeted to combat neurodegeneration during ALS pathophysiology.

Astrocytes in ALS

SOD1 models

Astrocytosis has been found in SOD1^{G37R} mice before onset of a motor phenotype, though it is unclear if this precedes motor neuron degeneration, and it increases during disease progression[75,110]. Astrocyte-restricted expression of SOD1^{G86R} in transgenic mice resulted in astrocytosis, but no ALS disease phenotype was evident[111]. Conversely, selective deletion of SOD1^{G85R} from astrocytes delayed disease onset and prolonged early disease progression[112], while SOD1^{G37R} deletion did not affect disease onset but significantly slowed later disease progression[110]. In keeping with these findings, transplantation of wild-type glial precursors that differentiated into astrocytes in the spinal cord of SOD1^{G93A} rats prolonged survival and disease duration and attenuated motor neuron degeneration[113], while transplanted SOD1^{G93A} astrocyte precursors induced motor neuron death and motor dysfunction in wild-type rats[114]. These data are consistent with the effect of microglia in SOD1 models, indicating that astrocyte activation contributes to neurotoxicity in disease pathogenesis.

In co-culture experiments, the expression of different forms of mutant SOD1 in astrocytes led to the death of primary and mouse stem-cell derived motor neurons via the release of soluble factors the identity of which is not yet determined, and this effect was more severe when co-cultured with mutant SOD1-expressing motor neurons[115-119]. In contrast, knockdown of mutant SOD1 attenuated astrocyte-mediated toxicity towards motor neurons[119]. Pathways involved in mutant SOD1-expressing astrocytes include dysregulated pro-nerve growth factor release, increased expression and secretion of molecules related to oxidative stress, dysregulated TGF-β signaling, and elevated pro-inflammatory gene expression [118,120-126] (FIG. 1). Recently, using a combination of bioinformatic prediction models and experimental validation, it was shown that one of the mechanisms by which mutant SOD1^{G93A}-expressing astrocytes kill wild-type motor neurons is amyloid precursor protein (APP) fragment release and activation of death-

receptor-6 (DR6) on motor neurons, which is prevented by DR6 knock-out and knockdown[127]. These findings suggest that expression of mutant SOD1 in astrocytes leads to gain-of-function non-cell-autonomous neurotoxicity. Loss-of-function effects in mSOD1-expressing astrocytes have also been described, for instance, a reduction in their homeostatic function in lactate provision, altered release of neurotrophic factors, and attenuated protection for neurons from glutamate-induced excitotoxicity[68,121,124,128,129] (FIG. 1). As a proof of concept, restoring these functions improves motor neuron survival[68].

Together, these results indicate that astrocytes can, similar to microglia, adopt neurotoxic properties in SOD1 models, which, in concert with mutant SOD1-induced primary neuronal damage, play an active role in disease pathogenesis. Recently, the crosstalk between astrocytes and other non-neuronal cells has been studied in more detail. Microglia were found to induce the conversion of a quiescent astrocyte to a pro-inflammatory A1 phenotype by the release of IL-1α, TNFα, and C1q[42] (FIG. 1). Triple knock-out of these factors led to a drastic reduction in the number of reactive astrocytes in $SOD1^{G93A}$ mice, improving life span, and delaying motor neuron loss and disease progression[126]. Conversely, upregulated TGF-β in astrocytes was reported to interfere with the neuroprotective function of microglia and T-cells in $SOD1^{G93A}$ mice, thereby accelerating disease progression[125]. Interestingly, the transplantation of $SOD1^{G93A}$ astrocytes also leads to the activation of microglia, the blockage of which partly rescued motor neuron loss[114], and microglial activation was delayed by deletion of $SOD1^{G37R}$ or $SOD1^{G85R}$ from astrocytes[110,112]. Hence, there is emerging evidence for a pro-inflammatory astrocytic phenotype in SOD1-ALS, influenced by reciprocal interaction between astrocytes and other non-neuronal cells, but a detailed and temporal analysis over the disease course remains to be described, particularly with regards to the crosstalk with microglia. Bearing in mind the expression of anti-inflammatory or neuroprotective markers in microglia reported at

early disease stages in SOD1 models, future studies should also evaluate potential neuroprotective properties of astrocytes in more detail.

TDP-43 models

Motor neuron-selective reduction of TDP-43^{Q331K} in transgenic mice delays disease onset but does not alter disease progression or prevent astrogliosis[130], indicating a contribution to pathogenesis for astrocytes also in TDP-43 models. Astrocyte-restricted expression of TDP-43^{M337V} in rats leads to astrogliosis causing progressive non-cell-autonomous motor neuron loss and paralysis, accompanied by astrocytic depletion of glutamate transporters, induction of the neurotoxic factor Lipocalin-2, and microglial activation[131] (FIG. 1). Dysregulated astrocytic metabolism, possibly leading to impaired provision of lactate to neurons, has also been observed in primary rat astrocytes upon expression of inclusion-forming C-terminal fragments of TDP-43[132]. In addition, primary astrocytes from TDP-43^{Q331K}-expressing mice show decreased levels of the anti-oxidant molecule glutathione and, similarly, astrocyte-conditioned medium from TDP43^{A315T}-expressing mice causes primary rat motor neuron death in a nitroxidative stress-dependent manner[133,134] (FIG. 1). A detailed assessment of TDP-43^{M337V}-expressing primary rat astrocytes and the spinal cord of transgenic rats revealed the downregulation of neurotrophic and upregulation of neurotoxic genes, such as Lipocalin-2 and Chi3L1[135]. Lipocalin-2 has also been identified as a neurotoxic factor released from astrocytes in organotypic cultures of brain slices from rats with selective TDP-43^{M337V}-expression in forebrain neurons and in the spinal cord of transgenic rats with motor neuron-specific TDP-43^{M337V}-expression, and its levels increased with progressive neurodegeneration[136].

While these findings were observed with overexpression of TDP-43, similar results have been associated with reduced TDP-43 levels. Conditional motor neuron-specific TDP-43 knock-out mice develop age-dependent progressive motor impairment and motor neuron degeneration,

and show progressive astrogliosis in the spinal cord[137,138]. In transgenic mice with RNAi-mediated TDP-43 knockdown, where TDP-43 levels appeared to be preferentially reduced in astrocytes rather than motor neurons in the spinal cord, degeneration of motor neurons, motor weakness and paralysis were observed[139]. Similarly, knock-down of TDP-43 in primary rat astrocytes led to the activation of pro-inflammatory mediators, including Lipocalin-2[140]. Collectively, these findings point to a crucial effect of TDP-43 dysfunction in astrocytes, with both TDP-43 mutations and depletion leading to neurotoxic properties. However, TDP-43 knockdown is unlikely to accurately model every aspect of TDP-43 dysfunction in ALS. TDP-43 deletion is embryonically lethal[141], while postnatal TDP-43 deletion also results in rapid death[142], indicating that TDP-43 levels are tightly regulated and play an essential role in cells. Phenotypes resulting from TDP-43 knockdown might therefore be merely the consequence of altering the levels of a crucial constitutively expressed protein.

In contrast, co-culture of iPSC-derived astrocytes expressing TDP-43^{M337V} with wild-type and TDP-43^{M337V}-expressing neurons did not affect neuronal survival[143]. Similarly, primary astrocytes overexpressing TDP-43^{A315T} or lacking TDP-43 were not toxic to wild-type motor neurons in co-culture or after transplantation of their precursors into the spinal cord of wild-type mice[144]. In co-culture and conditioned media paradigms, iPSC-derived control astrocytes had a protective effect against seeded aggregation of TDP-43 in iPSC-derived control motor neurons by reducing TDP-43 mislocalization and cell death[145]. However, whether control astrocytes that do not express mutant TDP-43 or TDP-43 aggregates represent an accurate disease model is questionable.

Together, the majority of currently available evidence points to both gain- and loss-of-function toxicity of astrocytes towards neurons with alterations in TDP-43, in line with the results from SOD1 models. Divergent findings might be due to different expression levels of TDP-43[124],

lack of modelling crosstalk with microglia and other non-neuronal cells, or differences between rodent and human microglia. Further studies using mutant TDP-43 iPSC-derived astrocytes are warranted to provide clarification.

C9orf72 models

Expression of G_4C_2 repeats and poly(GA) in the mouse CNS leads to astrogliosis, which is suggested to precede neurodegeneration[146-148]. Astrocytes directly differentiated from *C9orf72* fibroblasts (so-called induced, or i-astrocytes) cause significant cell death of co-cultured mouse embryonic stem-cell derived motor neurons, likely through a toxic gain-of-function mechanism[149,150]. Depletion of SRSF1, a nuclear export adaptor for the transport of *C9orf72* transcripts leading to reduced cytoplasmic RNA foci in these fibroblast-derived astrocytes, rescued motor neuron death, directly linking the *C9orf72* expansion with the adoption of neurotoxic properties in astrocytes[150]. Mechanistically, iPSC-derived *C9orf72* astrocytes showed increased oxidative stress and reduced release of antioxidants, and their conditioned media reduced the viability of mouse cortical neurons and human embryonic stem cell-derived motor neurons, possibly via increased neuronal oxidative stress[151] (FIG. 1).

In contrast to direct toxicity, dysfunctional adenosine, fructose and glycogen metabolism has been reported in *C9orf72* i-astrocytes, associated with increased death of mouse motor neurons in co-culture[152,153]. Rescuing deficits in adenosine metabolism ameliorated this neurotoxicity in co-culture[152]. Similarly, altered glutamate content was found in iPSC-derived astrocytes carrying a *C9orf72* expansion, probably through a loss-of-function effect on astrocytes[154]. Extracellular vesicles (EVs) also appear to play a role in the neurotoxic phenotype of C9orf72 i-astrocytes, with miR-494-3p, a negative regulator of Semaphorin 3A and other targets, being downregulated, with rescuing its levels improving motor neuron survival and neurite outgrowth[155]. Broader roles for EVs in reflecting the pathology and potentially influencing

propagation of neurodegenerative disorders are recognised[156]. iPSC-derived C9orf72 astrocytes have also been associated with electrophysiological dysfunction when co-cultured with wild-type iPSC-derived motor neurons[157], but the detailed mechanism mediating toxicity was not explored in this study[157].

Taken together, there is quite clear evidence that astrocytes adopt neurotoxic properties in *C9orf72* models. Future studies are needed to shed more light on the detailed mechanisms through which gain- and loss-of-function mechanisms act in combination and should focus on further identification of secreted cytotoxic factors and contact-dependent mechanisms of astrocyte-dependent cytotoxicity.

Summary

Taken together, clear astrocytic activation is found in SOD1, TDP43, and C9orf72 models of ALS, and most studies across the three different model systems support a neurotoxic astrocyte phenotype. This neurotoxicity appears to be mediated by the release of neurotoxic factors and a failure of astrocytes in their supportive functions to neurons. In contrast to microglia, there is currently no evidence for a neuroprotective role of astrocytes at early stages of the disease course. There is clear crosstalk with other non-neuronal cells, and preliminary evidence suggests that astrocytes might be skewed to neurotoxicity by pro-inflammatory microglia, but further studies that model multicellular interaction are needed to disentangle the direct effect of ALS mutations on astrocytes and the pro-inflammatory contribution of other cells to astrocyte toxicity.

Oligodendrocytes and NG2-glia in ALS

SOD1 models

Degeneration and loss of oligodendrocytes, but constant overall oligodendrocyte numbers due to increased oligodendrocyte precursor NG2 glia proliferation, with incomplete and failed differentiation resulting in progressive demyelination, have been found in the spinal cord of SOD^{G93A} transgenic mice before disease onset[158,159]. Selective Cre-mediated removal of $SOD1^{G37R}$ from NG2-glia delayed disease onset and prolonged survival, indicating a detrimental role of oligodendroglia in SOD1 models[158]. Interestingly, this also led to delayed astrocytic and microglial activation. Motor neuron degeneration in mice expressing $SOD1^{G37R}$ in motor neurons and oligodendrocytes can be mitigated by the proximity of other cells not expressing mutant SOD1 in chimeric mice, arguing that cells other than oligodendrocytes contribute to neurodegeneration[160]. However, primary mouse oligodendrocytes expressing $SOD1^{G93A}$ directly caused wild-type mouse motor neuron hyperexcitability and death, and similar motor neuron toxicity was observed in co-culture with iPSC- and induced neural progenitor cell-derived oligodendrocytes from sporadic, $SOD1^{D90A}$ and other familial (TDP-43^{G298S} and C9orf72) ALS patients[161].

Reduced levels of the monocarboxylate transporter 1, MCT1, have been found in the spinal cord of $SOD1^{G93A}$ transgenic mice and zebrafish, and *post mortem* tissue of ALS patients[159,162,163]. Deletion of $SOD1^{G93A}$ from NG2-glia restored MCT1 levels, indicating that it may cause neurotoxicity by impairing oligodendroglial support mechanisms[158] (FIG. 1). Furthermore, impaired lactate production and release, and reduced MCT1 levels, were found in fibroblast-derived oligodendrocytes from the SOD^{G93A} mouse and patient carrying the recessive human SOD1 D90A ALS mutation[161]. While restoring lactate levels fully rescued toxicity when $SOD1^{G93A}$ oligodendrocyte conditioned medium was added to motor neurons, it only partly rescued decreased motor neuron survival in co-culture, arguing for additional close contact-

dependent mechanisms[161]. Although SOD1^{G93A} knock-down in NG2-glia efficiently rescued motor neuron death and elevated lactate levels, it did not modulate the toxicity exerted by differentiated oligodendrocytes, reinforcing a role for early dysfunction in the oligodendroglial lineage in SOD1-ALS[161]. Translating ribosome affinity purification and high-throughput RNA sequencing of SOD1^{G37R} transgenic mice identified early dysregulation in motor neurons followed by changes in astrocytes and oligodendrocytes[164]. In oligodendrocytes, changes became obvious at an early symptomatic disease stage, with no changes found in the gene expression of *MCT1* but membrane and lipid signaling defects with an upregulation of ETS transcription factors instead[164].

Collectively, there is clear evidence from SOD1 models implicating early oligodendrocyte dysfunction in disease pathogenesis, primarily via reduced metabolic support to neurons. Additional mechanisms of toxicity are likely to play an additional role and should be investigated in future studies. In particular, the interplay between oligodendrocytes and other glial cells also remains to be characterized in more detail.

TDP-43 models

Research into the role of oligodendrocytes in TDP-43 models is limited. TDP43^{G298S} fibroblast-derived oligodendrocytes caused motor neuron death in co-culture, via reduced lactate release, similar to mutant SOD1-derived oligodendrocytes[161]. Future studies will be required to further elucidate the role of TDP-43 aggregates and mutations on oligodendrocytic function.

C9orf72 models

Oligodendrocytes have also been successfully derived from patients carrying the *C9orf72* expansion[161,165], but knowledge of their functional role is limited. In iPSC-derived

oligodendrocytes, RNA foci but no DPR formation were observed, with no defects in maturation apparent[165]. Induced neural precursor-derived oligodendrocytes from *C9orf72* expansion-associated ALS patients caused motor neuron toxicity in co-culture[161]. However, in contrast to mutations in *SOD1* and *TARDBP*, this was not mediated via reduced lactate release, although soluble factors likely played a role[161]. Further studies are needed to characterize the effect of the *C9orf72* expansion on oligodendrocytes and their non-cell-autonomous influence on motor neurons in more detail.

Summary

Most evidence for the role of oligodendrocytes in experimental models of ALS comes from SOD1 models, and there is a clear need for more studies on their role in TDP-43 and C9orf72 models. However, currently available evidence supports a neurotoxic effect of oligodendrocytes, which occurs early and is connected to a failure in their maturation from NG2 glial precursors. Reduced metabolic support of neurons through lower lactate release seems to be a key mechanism of oligodendroglial toxicity, but the elucidation of additional means of toxicity in crosstalk with other non-neuronal cells in future studies is warranted.

Non-glial non-neuronal cells in ALS

Monocytes and macrophages in SOD1, TDP-43, and C9orf72 models

In $SOD1^{G93A}$ mice, increased expression of chemotactic genes, such as monocyte chemoattractant protein-1 (*MCP-1/CCL2*), was found in microglia, neurons and other cells, which is thought to lead to the recruitment of peripheral monocytes to the spinal cord[166] (FIG. 2). These monocytes show a pro-inflammatory profile prior to disease onset, and targeting them using i.p. Ly6C antibody treatment skewed them to an anti-inflammatory phenotype, delayed disease onset, extended survival, and attenuated neuronal loss[166]. It is noteworthy that the

antibody was peripherally injected in this model, so it is difficult to disentangle whether these beneficial effects were mainly due to the reduced monocyte infiltration observed by the authors or a general immunosuppressive effect at the periphery, as monocytes extracted from the blood of ALS patients and monocyte-derived macrophages have a pro-inflammatory profile[166-168]. Conversely, a neuroprotective role of monocyte-derived cells was indicated by a correlation between the number of surviving motor neurons and the presence of peripheral myeloid cells in the spinal cord at early disease stages of SOD1^{G93A} transgenic mice[169]. Furthermore, promoting invasion of monocytes using human immunoglobulins or fusion proteins was associated with increased motor neuron survival and delayed disease onset in this study[169]. These apparently contradictory findings might be resolved by a model similar to the *early* neuro*protective*, but *late* neuro*toxic* properties reported for microglia in SOD1 models. However, co-culture studies of neurons and macrophages are warranted to provide more insight.

Numerous infiltrating monocytes were also observed within and around the blood vessels in the brain of a TDP-43^{A315T} mouse model and in ALS patients with TDP-43 pathology[86], but an assessment of monocyte/macrophage function in TDP-43 models remains incomplete. In models based on *C9orf72*, loss-of-function results in a pro-inflammatory state in peripheral monocytes and macrophages, with dysfunctional lysosomal trafficking and autophagy impairment[93,170]. Recently, this was demonstrated to result in the STING-mediated hyperactivation of interferon signaling[170], thereby indicating potential neurotoxic properties. Results of experiments investigating *C9orf72* gain-of-function toxicity are yet to be published.

Important physiological roles of CNS-associated macrophages are beginning to emerge, including evidence for a pathophysiological role in AD[171]. To shed further light on the pathophysiological role of peripheral monocytes/macrophages in ALS and their effects on neurons, iPSC-derived macrophages might prove a useful tool.

T-cells in SOD1, TDP-43, and C9orf72 models

In SOD1^{G93A} mice, both CD4^{+} and CD8^{+} T-cells infiltrate the spinal cord during disease progression[62,172,173]. Lack of functional T-cells or CD4^{+} T-cells increased pro-inflammatory mediator release from microglia and astrocytes, decreased the levels of anti-inflammatory and trophic factors, and accelerated disease progression[172,173]. Reconstitution with bone marrow transplants rescued these deficits, and shifted microglia and astrocytes to a neuroprotective phenotype[173], indicating a neuroprotective role of T-cells in SOD1-ALS. Closer evaluation of the CD4^{+} T-cell population revealed that SOD1^{G93A}-expressing regulatory T-cells (T-reg) are increased at early slowly progressing disease stages, which induce a neuroprotective M2 microglia phenotype via IL-4 release and suppress pro-inflammatory Th1-cells, while at later, fast-progressing stages, their levels decrease or they become dysfunctional, and Th1-cells and M1 microglia predominate[174-176] (FIG. 2). In agreement, expansion of T-reg cells in SOD1^{G93A}-expressing mice reduced microglial and astrocytic activation, preserved motor neuron size and prolonged survival. Decreased T-reg numbers or greater dysfunction in the blood of human patients were both associated with faster rates of disease progression[174,176-178]. It is not yet clear how this switch in T-cell properties between early and later stages is mediated.

CD8^{+} T-cells, on the other hand, have been relatively understudied, although CD8^{+} T-cells are one of the major infiltrating populations in the spinal cord of SOD1^{G93A} mice[179]. β2 microglobulin-deficient SOD1^{G93A} mice, which lack MHC1 and are defective for mature CD8^{+} T-cells, showed delayed forelimb paralysis and prolonged survival[180]. Similarly, SOD1^{G93A} mice with CD8a knockout, which do not generate functional CD8^{+} T cells, showed no effects on disease onset or motor performance but increased spinal motor neuron survival[181]. However, i.p. injection of an anti-CD8 antibody partially eliminated CD8^{+} T-cell infiltration but had no effect on survival in one study[179], or had a less pronounced effect on CD8^{+} T cell infiltration

and motor neuron survival in a different study[181]. The authors of the latter study hypothesized that the lower protective effect of the antibody treatment might be due to the preferential action of the antibody on naïve rather than infiltrating $CD8^+$ T cells. Importantly, in co-culture, $SOD1^{G93A}$-expressing $CD8^+$ T-cells caused wild-type and $SOD1^{G93A}$-expressing motor neuron death, through interferon-γ release alongside Fas and granzyme death pathway-mediated mechanisms[181] (FIG. 2).

Evidence from SOD1 models outlines a neuroprotective role of $CD4^+$ T-cells in crosstalk with microglia and astrocytes, the failure of which, together with $CD8^+$ T-cell activation, leads to neurotoxicity. Evidence about the role of T-cells in other experimental models of ALS is scarce. In *C9orf72*-deficient mice, T-cell activation has been demonstrated[170,182], but their functional role remains to be determined, particularly in the context of gain-of-toxicity hypotheses. In a recent study, iPSC-derived M2 macrophages from *C9orf72* expansion and sporadic ALS patients induced and sustained anti-inflammatory Treg function, while pro-inflammatory M1 macrophages and cytotoxic T-cells were suppressed[183]. Hence, it would be of interest to explore the effects of these cells on microglial and astrocytic function, in particular in crosstalk with motor neurons, and to see if neuroprotective effects might also be observed *in vivo*. Further studies employing multi-cellular models using patient-derived T-cells are warranted to shed more light on their functional role in ALS pathogenesis.

NK cells in SOD1, TDP-43, and C9orf72 models

Aberrant numbers of NK cells have been found in the peripheral blood and CNS of ALS patients, and $SOD1^{G93A}$-expressing mice display a high number of NK cells in the spinal cord[62,172,184,185]. Recruitment of NK cells to the CNS was demonstrated to be dependent on CCL2[62] (FIG. 2). Downregulation of NK cell activity led to the recruitment of T-cells into the spinal cord before the onset of clinical symptoms, attenuated motor neuron loss, reduced

astrogliosis, delayed disease onset and improved lifespan[184]. While anti-NK1.1 antibody-mediated reduction of NK cell infiltration into the spinal cord delayed motor neuron impairment and increased survival in TDP43^{A315T}-expressing mice and additionally attenuated early motor neuron loss in SOD1^{G93A} transgenic mice[62], it had no effect on survival when administered at later disease stages[62,179], indicating an early role of NK cells in the pathophysiology of SOD1 ALS models. Both wild-type and SOD1^{G93A}-expressing NK cells were neurotoxic in co-culture, but only to SOD1^{G93A}-expressing motor neurons. This effect was dependent on the release of toxic factors including perforin and contact-dependent mechanisms, such as the expression of NKG2D ligands on motor neurons[62] (FIG. 2). Furthermore, NK cell depletion induced an anti-inflammatory microglial phenotype, and increased the number of T-reg cells in the spinal cord via reduced interferon-γ release[62] (FIG. 2). Together, there is initial evidence for a neurotoxic role of NK cells in SOD1-ALS, in crosstalk with T-cells and microglia. Additional studies, also including mutant TDP-43 and *C9orf72* expansion-based models of ALS, will be required to provide more insight into their functional role in ALS pathogenesis.

Biomarkers from non-neuronal cells

Non-neuronal cell-derived molecules can be detected and measured in the CSF or peripheral blood of human ALS patients. Their levels, particularly if measured longitudinally, provide a window into the pathogenic role of non-neuronal cells in humans, and their prognostic and pharmacodynamic biomarker potential is increasingly being evaluated. While there is a growing list of potential candidates, the focus here is on the most promising biochemical markers associated with non-neuronal cells, based on the consistency of their upregulation in the CSF of ALS patients across multiple different studies and laboratories (for comprehensive recent ALS biomarker reviews including other biomarker candidates, see[186,187]).

Multiple studies have demonstrated increased abundance of chitotriosidase-1 (CHIT1), chitinase-3-like protein 1 (CHI3L1), and chitinase-3-like protein 2 (CHI3L2) in the CSF of ALS patients[10,188-196]. Their CSF levels correlate with disease progression rate and phosphorylated neurofilament heavy chain as a proxy for axonal damage[10]. While CHIT1 is an enzymatically active hydrolase, CHI3L1 and CHI3l2 possess no catalytic motif[197]. Their release could be due to the potential presence of chitin-like polymers in ALS brains, as observed in amyloid plaques in AD[198], or because of a newly acquired but thus far unknown role in the human immune system.

CHIT1 is expressed in macrophages and microglia[188,189]. CSF CHIT1 levels are higher in ALS patients compared to other neurodegenerative disorders, and are associated with shortened survival and higher disease progression rate[10,189,191,194,199]. In a longitudinal assessment of phenoconverters, CSF CHIT1 levels and activity were relatively low in the pre-symptomatic period but showed a sharp increase between the late pre-symptomatic and early symptomatic phase[192,193]. A recent study found that intrathecal injection of CHIT1 into rats was associated with increased microglial numbers and activation, astrogliosis, release of pro-inflammatory cytokines and motor neuron loss[190]. These studies provide emerging evidence for an important microglial/macrophage role in the disease course of human ALS patients, and, in line with experimental models, probably rather as a secondary toxic response to damage than a primary disease driver. They also underscore the usefulness of CHIT1 as a biomarker best suited to disease monitoring and the potential evaluation of a response to treatment. Insights into its cellular role, however, remain to be identified. Future studies will be required to uncover the trigger for CHIT1 release, its cellular and molecular targets, and its cellular expression at different stages of the disease.

CHI3L1 appears to be predominantly expressed in astrocytes in ALS patients[192,199,200]. CSF CHI3L1 levels increase over time, correlate with upper motor neuron symptoms and cognitive impairment, and showed an inverse correlation with patient survival[10,191,194,195,200]. In asymptomatic mutation carriers, CHI3L1 levels were unchanged compared to healthy control individuals, and in contrast to CHIT1, the longitudinal assessment showed no striking difference between the late pre-symptomatic and early symptomatic phase[192,193]. Overexpression of TDP-43^{M337V} in primary astrocytes led to the upregulation of CHI3L1, and administration of synthetic CHI3L1 induced primary neuronal death[135]. While this evidence for CHI3L1 release corroborates the activation and putative neurotoxic role of astrocytes observed in experimental models of ALS, it argues against a pronounced pathogenic function during the early disease course. Future studies will need to elucidate the trigger for CHI3L1 release and its molecular targets in more detail. However, another astrocyte-depended biomarker for ALS that is associated with early pathology has recently been described in patients; the levels of IL-6 in astrocyte-derived exosomes from the blood of people with sporadic type ALS are increased and positively associated with the rate of disease progression, but only in those with a disease duration of less than 12 months[201].

CHI3L2 has not been extensively studied, but its expression has been observed in macrophages[10,197] and CSF levels correlate with disease progression rate[10,191]. Similar to CHI3L1, CHI3L2 levels were not raised in asymptomatic mutation carriers and the longitudinal assessment showed no striking difference between the late presymptomatic and early symptomatic phase[192]. Due to the lack of mechanistic studies, the biological meaning of CHI3L2 in crosstalk with neurons is currently uncertain.

In addition, monocyte chemoattractant protein-1 (MCP-1/CCL2) has been studied as a marker of non-neuronal cell involvement in ALS. In experimental models, MCP-1/CCL2 expression

in cells involved in ALS pathogenesis, including motor neurons, astrocytes, and microglia, is thought to result in the recruitment of CCR2-expressing cells, such as microglia, peripheral macrophages, T-cells, and NK cells[34,62,86,202] (FIG. 2). Elevated levels of MCP-1 have been reported in the CSF of ALS patients, and were inversely correlated with survival, but a correlation with the disease progression rate was not consistently found[194,203-208]. Its upregulation in human patients likely reflects the glial activation and recruitment of peripheral immune cells into the CNS observed in experimental models. Its biological meaning, however, is not clear, as both neurotoxic and neuroprotective properties of MCP-1-dependent cell infiltration have been demonstrated.

Conclusions and future directions

An important role of non-neuronal cells in ALS pathogenesis is now clear, and evidence from experimental models largely replicates the activation of glial cells and infiltration of peripheral immune cells found in neuroimaging, post mortem tissue, and biomarker studies in humans. Even though there is some inconsistency across the experimental models employed and SOD1 model-based evidence may have limited relevance to the majority of ALS that is associated with TDP-43 aggregates, a common theme is emerging across the different non-neuronal cell types and models that cell-autonomous neuronal dysfunction is exacerbated by non-cell-autonomous toxicity. Initial analyses of cell-cell-interactions in iPSC-derived human cells have already confirmed some of the key findings from rodent models. Primary neuronal damage is likely to initiate the onset of disease, while, over the disease course, the combination of a dysregulated and pro-inflammatory non-neuronal cell response to neuronal damage together with the primary failure of the supportive roles of non-neuronal cells influences progression. Particularly at early disease stages, however, microglia and regulatory T-cells exert some neuroprotection in experimental models. Differences in the specific mechanisms of non-

neuronal toxicity in experimental models for SOD1, TDP-43, and C9orf72 ALS argue in favour of therapeutic approaches targeted according to genotype, and may partially explain why multiple clinical trials non-specifically targeting the immune system have not proved successful thus far. However, several new therapeutics targeting non-neuronal cells are currently undergoing clinical trials (for a recent review on clinical trials, see[209]). For instance, masitinib, a tyrosine kinase inhibitor that targets microglial, macrophage, and mast cell activation, slowed disease progression in ALS patients in a phase 2/3 trial when used as an add-on to riluzole[210]. Future studies should include the differentiation of further non-neuronal cell types and also focus on the creation and usage of human-derived multi-cellular models to more accurately model the disease and crosstalk between cells found *in vivo*. More accurate disease models will facilitate drug screening in a more physiological environment. Improved understanding of the nuanced pathophysiological role of non-neuronal cells will guide the re-purposing of licensed drugs as well as novel development. Biomarkers derived from non-neuronal cells show promise for the future assessment of emerging therapeutic candidates.

References

1 Talbot, K., Feneberg, E., Scaber, J., Thompson, A. G. & Turner, M. R. Amyotrophic lateral sclerosis: the complex path to precision medicine. *J Neurol* **265**, 2454-2462 (2018).

2 Al-Chalabi, A., van den Berg, L. H. & Veldink, J. Gene discovery in amyotrophic lateral sclerosis: implications for clinical management. *Nat Rev Neurol* **13**, 96-104 (2017).

3 Renton, A. E., Chio, A. & Traynor, B. J. State of play in amyotrophic lateral sclerosis genetics. *Nature neuroscience* **17**, 17-23 (2014).

4 DeJesus-Hernandez, M. *et al.* Expanded GGGGCC hexanucleotide repeat in noncoding region of C9ORF72 causes chromosome 9p-linked FTD and ALS. *Neuron* **72**, 245-256 (2011).

5 Rosen, D. R. *et al.* Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature* **362**, 59-62 (1993).

6 Sreedharan, J. *et al.* TDP-43 mutations in familial and sporadic amyotrophic lateral sclerosis. *Science* **319**, 1668-1672 (2008).

7 Vance, C. *et al.* Mutations in FUS, an RNA processing protein, cause familial amyotrophic lateral sclerosis type 6. *Science (New York, N.Y.)* **323**, 1208-1211 (2009).

8 Neumann, M. *et al.* Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Science* **314**, 130-133 (2006).

9 Brown, R. H., Jr. & Al-Chalabi, A. Amyotrophic Lateral Sclerosis. *N Engl J Med* **377**, 1602 (2017).

10 Thompson, A. G. *et al.* Cerebrospinal fluid macrophage biomarkers in amyotrophic lateral sclerosis. *Ann Neurol* **83**, 258-268 (2018).

11 von Bartheld, C. S., Bahney, J. & Herculano-Houzel, S. The search for true numbers of neurons and glial cells in the human brain: A review of 150 years of cell counting. *J Comp Neurol* **524**, 3865-3895 (2016).

12 Jakel, S. & Dimou, L. Glial Cells and Their Function in the Adult Brain: A Journey through the History of Their Ablation. *Front Cell Neurosci* **11**, 24 (2017).

13 Allen, N. J. & Lyons, D. A. Glia as architects of central nervous system formation and function. *Science* **362**, 181-185 (2018).

14 Greenhalgh, A. D., David, S. & Bennett, F. C. Immune cell regulation of glia during CNS injury and disease. *Nat Rev Neurosci* **21**, 139-152 (2020).

15 Pelvig, D. P., Pakkenberg, H., Stark, A. K. & Pakkenberg, B. Neocortical glial cell numbers in human brains. *Neurobiol Aging* **29**, 1754-1762 (2008).

16 Alliot, F., Godin, I. & Pessac, B. Microglia derive from progenitors, originating from the yolk sac, and which proliferate in the brain. *Developmental Brain Research* **117**, 145-152 (1999).

17 Ginhoux, F. *et al.* Fate mapping analysis reveals that adult microglia derive from primitive macrophages. *Science* **330**, 841-845 (2010).

18 Bruttger, J. *et al.* Genetic Cell Ablation Reveals Clusters of Local Self-Renewing Microglia in the Mammalian Central Nervous System. *Immunity* **43**, 92-106 (2015).

19 Kettenmann, H., Hanisch, U. K., Noda, M. & Verkhratsky, A. Physiology of microglia. *Physiol Rev* **91**, 461-553 (2011).

20 Nimmerjahn, A., Kirchhoff, F. & Helmchen, F. Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. *Science* **308**, 1314-1318 (2005).

21 Li, Q. & Barres, B. A. Microglia and macrophages in brain homeostasis and disease. *Nat Rev Immunol* **18**, 225-242 (2018).

22 Butovsky, O. & Weiner, H. L. Microglial signatures and their role in health and disease. *Nat Rev Neurosci* **19**, 622-635 (2018).

23 Ransohoff, R. M. A polarizing question: do M1 and M2 microglia exist? *Nat Neurosci* **19**, 987-991 (2016).

24 Mantovani, A., Sica, A. & Locati, M. Macrophage polarization comes of age. *Immunity* **23**, 344-346 (2005).

25 Hu, X. *et al.* Microglial and macrophage polarization-new prospects for brain repair. *Nat Rev Neurol* **11**, 56-64 (2015).

26 Ashford, B. A. *et al.* Review: Microglia in motor neuron disease. *Neuropathol Appl Neurobiol* (2020).

27 Tang, Y. & Le, W. Differential Roles of M1 and M2 Microglia in Neurodegenerative Diseases. *Mol Neurobiol* **53**, 1181-1194 (2016).

28 Cherry, J. D., Olschowka, J. A. & O'Banion, M. K. Neuroinflammation and M2 microglia: the good, the bad, and the inflamed. *J Neuroinflammation* **11**, 98 (2014).

29 Vass, K. & Lassmann, H. Intrathecal application of interferon gamma. Progressive appearance of MHC antigens within the rat nervous system. *Am J Pathol* **137**, 789-800 (1990).

30 Colonna, M. & Butovsky, O. Microglia Function in the Central Nervous System During Health and Neurodegeneration. *Annu Rev Immunol* **35**, 441-468 (2017).

31 Turner, M. R. *et al.* Evidence of widespread cerebral microglial activation in amyotrophic lateral sclerosis: an [11C](R)-PK11195 positron emission tomography study. *Neurobiol Dis* **15**, 601-609 (2004).

32 Zurcher, N. R. *et al.* Increased in vivo glial activation in patients with amyotrophic lateral sclerosis: assessed with [(11)C]-PBR28. *Neuroimage Clin* **7**, 409-414 (2015).

33 Tondo, G. *et al.* (11) C-PK11195 PET-based molecular study of microglia activation in SOD1 amyotrophic lateral sclerosis. *Ann Clin Transl Neurol* (2020).

34 Henkel, J. S. *et al.* Presence of dendritic cells, MCP-1, and activated microglia/macrophages in amyotrophic lateral sclerosis spinal cord tissue. *Ann Neurol* **55**, 221-235 (2004).

35 Brettschneider, J. *et al.* Microglial activation correlates with disease progression and upper motor neuron clinical symptoms in amyotrophic lateral sclerosis. *PLoS One* **7**, e39216 (2012).

36 Brettschneider, J. *et al.* Microglial activation and TDP-43 pathology correlate with executive dysfunction in amyotrophic lateral sclerosis. *Acta Neuropathol* **123**, 395-407 (2012).

37 Verkhratsky, A. & Nedergaard, M. Physiology of Astroglia. *Physiol Rev* **98**, 239-389 (2018).

38 Kriegstein, A. & Alvarez-Buylla, A. The glial nature of embryonic and adult neural stem cells. *Annu Rev Neurosci* **32**, 149-184 (2009).

39 Vainchtein, I. D. & Molofsky, A. V. Astrocytes and Microglia: In Sickness and in Health. *Trends Neurosci* **43**, 144-154 (2020).

40 Zamanian, J. L. *et al.* Genomic analysis of reactive astrogliosis. *J Neurosci* **32**, 6391-6410 (2012).

41 Sofroniew, M. V. & Vinters, H. V. Astrocytes: biology and pathology. *Acta Neuropathol* **119**, 7-35 (2010).

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

43 Miller, S. J. Astrocyte Heterogeneity in the Adult Central Nervous System. *Front Cell Neurosci* **12**, 401 (2018).

44 Clarke, L. E. *et al.* Normal aging induces A1-like astrocyte reactivity. *Proc Natl Acad Sci U S A* **115**, E1896-E1905 (2018).

45 Liddelow, S. A. & Barres, B. A. Reactive Astrocytes: Production, Function, and Therapeutic Potential. *Immunity* **46**, 957-967 (2017).

46 Kawamata, T., Akiyama, H., Yamada, T. & McGeer, P. L. Immunologic reactions in amyotrophic lateral sclerosis brain and spinal cord tissue. *Am J Pathol* **140**, 691-707 (1992).
47 Tam, O. H. *et al.* Postmortem Cortex Samples Identify Distinct Molecular Subtypes of ALS: Retrotransposon Activation, Oxidative Stress, and Activated Glia. *Cell Rep* **29**, 1164-1177 e1165 (2019).
48 Schiffer, D., Cordera, S., Cavalla, P. & Migheli, A. Reactive astrogliosis of the spinal cord in amyotrophic lateral sclerosis. *J Neurol Sci* **139 Suppl**, 27-33 (1996).
49 Nagy, D., Kato, T. & Kushner, P. D. Reactive astrocytes are widespread in the cortical gray matter of amyotrophic lateral sclerosis. *J Neurosci Res* **38**, 336-347 (1994).
50 Kushner, P. D., Stephenson, D. T. & Wright, S. Reactive astrogliosis is widespread in the subcortical white matter of amyotrophic lateral sclerosis brain. *J Neuropathol Exp Neurol* **50**, 263-277 (1991).
51 Johansson, A. *et al.* Evidence for astrocytosis in ALS demonstrated by [11C](L)-deprenyl-D2 PET. *J Neurol Sci* **255**, 17-22 (2007).
52 Valerio-Gomes, B., Guimaraes, D. M., Szczupak, D. & Lent, R. The Absolute Number of Oligodendrocytes in the Adult Mouse Brain. *Front Neuroanat* **12**, 90 (2018).
53 Bergles, D. E. & Richardson, W. D. Oligodendrocyte Development and Plasticity. *Cold Spring Harb Perspect Biol* **8**, a020453 (2015).
54 Philips, T. & Rothstein, J. D. Oligodendroglia: metabolic supporters of neurons. *J Clin Invest* **127**, 3271-3280 (2017).
55 Michalski, J. P. & Kothary, R. Oligodendrocytes in a Nutshell. *Front Cell Neurosci* **9**, 340 (2015).
56 Zhang, S. Z. *et al.* NG2 glia regulate brain innate immunity via TGF-beta2/TGFBR2 axis. *BMC Med* **17**, 204 (2019).
57 Nolan, M. *et al.* Quantitative patterns of motor cortex proteinopathy across ALS genotypes. *Acta Neuropathol Commun* **8**, 98 (2020).
58 Kierdorf, K., Masuda, T., Jordao, M. J. C. & Prinz, M. Macrophages at CNS interfaces: ontogeny and function in health and disease. *Nat Rev Neurosci* **20**, 547-562 (2019).
59 Prinz, M., Erny, D. & Hagemeyer, N. Ontogeny and homeostasis of CNS myeloid cells. *Nat Immunol* **18**, 385-392 (2017).
60 Galea, I. *et al.* An antigen-specific pathway for CD8 T cells across the blood-brain barrier. *J Exp Med* **204**, 2023-2030 (2007).
61 Engelhardt, B., Vajkoczy, P. & Weller, R. O. The movers and shapers in immune privilege of the CNS. *Nat Immunol* **18**, 123-131 (2017).
62 Garofalo, S. *et al.* Natural killer cells modulate motor neuron-immune cell cross talk in models of Amyotrophic Lateral Sclerosis. *Nat Commun* **11**, 1773 (2020).
63 Gurney, M. E. *et al.* Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation. *Science* **264**, 1772-1775 (1994).
64 Philips, T. & Rothstein, J. D. Rodent Models of Amyotrophic Lateral Sclerosis. *Curr Protoc Pharmacol* **69**, 5 67 61-65 67 21 (2015).
65 Turner, B. J. & Talbot, K. Transgenics, toxicity and therapeutics in rodent models of mutant SOD1-mediated familial ALS. *Prog Neurobiol* **85**, 94-134 (2008).
66 Hall, E. D., Oostveen, J. A. & Gurney, M. E. Relationship of microglial and astrocytic activation to disease onset and progression in a transgenic model of familial ALS. *Glia* **23**, 249-256 (1998).
67 Alexianu, M. E., Kozovska, M. & Appel, S. H. Immune reactivity in a mouse model of familial ALS correlates with disease progression. *Neurology* **57**, 1282-1289 (2001).
68 Philips, T. & Robberecht, W. Neuroinflammation in amyotrophic lateral sclerosis: role of glial activation in motor neuron disease. *Lancet Neurol* **10**, 253-263 (2011).

69 Pramatarova, A., Laganière, J., Roussel, J., Brisebois, K. & Rouleau, G. A. Neuron-Specific Expression of Mutant Superoxide Dismutase 1 in Transgenic Mice Does Not Lead to Motor Impairment. *The Journal of Neuroscience* **21**, 3369-3374 (2001).
70 Lino, M. M., Schneider, C. & Caroni, P. Accumulation of SOD1 Mutants in Postnatal Motoneurons Does Not Cause Motoneuron Pathology or Motoneuron Disease. *The Journal of Neuroscience* **22**, 4825-4832 (2002).
71 Wang, L. *et al.* Restricted expression of mutant SOD1 in spinal motor neurons and interneurons induces motor neuron pathology. *Neurobiol Dis* **29**, 400-408 (2008).
72 Jaarsma, D., Teuling, E., Haasdijk, E. D., De Zeeuw, C. I. & Hoogenraad, C. C. Neuron-specific expression of mutant superoxide dismutase is sufficient to induce amyotrophic lateral sclerosis in transgenic mice. *J Neurosci* **28**, 2075-2088 (2008).
73 Beers, D. R. *et al.* Wild-type microglia extend survival in PU.1 knockout mice with familial amyotrophic lateral sclerosis. *Proc Natl Acad Sci U S A* **103**, 16021-16026 (2006).
74 Ralph, G. S. *et al.* Silencing mutant SOD1 using RNAi protects against neurodegeneration and extends survival in an ALS model. *Nat Med* **11**, 429-433 (2005).
75 Boillee, S. *et al.* Onset and progression in inherited ALS determined by motor neurons and microglia. *Science* **312**, 1389-1392 (2006).
76 Liao, B., Zhao, W., Beers, D. R., Henkel, J. S. & Appel, S. H. Transformation from a neuroprotective to a neurotoxic microglial phenotype in a mouse model of ALS. *Exp Neurol* **237**, 147-152 (2012).
77 Gravel, M. *et al.* IL-10 Controls Early Microglial Phenotypes and Disease Onset in ALS Caused by Misfolded Superoxide Dismutase 1. *J Neurosci* **36**, 1031-1048 (2016).
78 Xiao, Q. *et al.* Mutant SOD1(G93A) microglia are more neurotoxic relative to wild-type microglia. *J Neurochem* **102**, 2008-2019 (2007).
79 Zhao, W. *et al.* Extracellular mutant SOD1 induces microglial-mediated motoneuron injury. *Glia* **58**, 231-243 (2010).
80 Weydt, P., Yuen, E. C., Ransom, B. R. & Moller, T. Increased cytotoxic potential of microglia from ALS-transgenic mice. *Glia* **48**, 179-182 (2004).
81 Gowing, G., Dequen, F., Soucy, G. & Julien, J. P. Absence of tumor necrosis factor-alpha does not affect motor neuron disease caused by superoxide dismutase 1 mutations. *J Neurosci* **26**, 11397-11402 (2006).
82 Meissner, F., Molawi, K. & Zychlinsky, A. Mutant superoxide dismutase 1-induced IL-1beta accelerates ALS pathogenesis. *Proc Natl Acad Sci U S A* **107**, 13046-13050 (2010).
83 Nguyen, M. D., Julien, J. P. & Rivest, S. Induction of proinflammatory molecules in mice with amyotrophic lateral sclerosis: no requirement for proapoptotic interleukin-1beta in neurodegeneration. *Ann Neurol* **50**, 630-639 (2001).
84 Chiu, I. M. *et al.* A neurodegeneration-specific gene-expression signature of acutely isolated microglia from an amyotrophic lateral sclerosis mouse model. *Cell Rep* **4**, 385-401 (2013).
85 Wegorzewska, I., Bell, S., Cairns, N. J., Miller, T. M. & Baloh, R. H. TDP-43 mutant transgenic mice develop features of ALS and frontotemporal lobar degeneration. *Proc Natl Acad Sci U S A* **106**, 18809-18814 (2009).
86 Jara, J. H. *et al.* MCP1-CCR2 and neuroinflammation in the ALS motor cortex with TDP-43 pathology. *J Neuroinflammation* **16**, 196 (2019).
87 Zhao, W. *et al.* TDP-43 activates microglia through NF-kappaB and NLRP3 inflammasome. *Exp Neurol* **273**, 24-35 (2015).
88 Deora, V. *et al.* The microglial NLRP3 inflammasome is activated by amyotrophic lateral sclerosis proteins. *Glia* **68**, 407-421 (2020).

89 Leal-Lasarte, M. M., Franco, J. M., Labrador-Garrido, A., Pozo, D. & Roodveldt, C. Extracellular TDP-43 aggregates target MAPK/MAK/MRK overlapping kinase (MOK) and trigger caspase-3/IL-18 signaling in microglia. *FASEB J* **31**, 2797-2816 (2017).
90 Spiller, K. J. *et al.* Microglia-mediated recovery from ALS-relevant motor neuron degeneration in a mouse model of TDP-43 proteinopathy. *Nat Neurosci* **21**, 329-340 (2018).
91 Svahn, A. J. *et al.* Nucleo-cytoplasmic transport of TDP-43 studied in real time: impaired microglia function leads to axonal spreading of TDP-43 in degenerating motor neurons. *Acta Neuropathol* **136**, 445-459 (2018).
92 Paolicelli, R. C. *et al.* TDP-43 Depletion in Microglia Promotes Amyloid Clearance but Also Induces Synapse Loss. *Neuron* **95**, 297-308 e296 (2017).
93 O'Rourke, J. G. *et al.* C9orf72 is required for proper macrophage and microglial function in mice. *Science* **351**, 1324-1329 (2016).
94 Lall, D. & Baloh, R. H. Microglia and C9orf72 in neuroinflammation and ALS and frontotemporal dementia. *J Clin Invest* **127**, 3250-3258 (2017).
95 O'Rourke, J. G. *et al.* C9orf72 BAC Transgenic Mice Display Typical Pathologic Features of ALS/FTD. *Neuron* **88**, 892-901 (2015).
96 Jiang, J. *et al.* Gain of Toxicity from ALS/FTD-Linked Repeat Expansions in C9ORF72 Is Alleviated by Antisense Oligonucleotides Targeting GGGGCC-Containing RNAs. *Neuron* **90**, 535-550 (2016).
97 Peters, O. M. *et al.* Human C9ORF72 Hexanucleotide Expansion Reproduces RNA Foci and Dipeptide Repeat Proteins but Not Neurodegeneration in BAC Transgenic Mice. *Neuron* **88**, 902-909 (2015).
98 Mackenzie, I. R. *et al.* Quantitative analysis and clinico-pathological correlations of different dipeptide repeat protein pathologies in C9ORF72 mutation carriers. *Acta Neuropathol* **130**, 845-861 (2015).
99 Zhou, Q. *et al.* Active poly-GA vaccination prevents microglia activation and motor deficits in a C9orf72 mouse model. *EMBO Mol Med* **12**, e10919 (2020).
100 Schludi, M. H. *et al.* Spinal poly-GA inclusions in a C9orf72 mouse model trigger motor deficits and inflammation without neuron loss. *Acta Neuropathol* **134**, 241-254 (2017).
101 LaClair, K. D. *et al.* Congenic expression of poly-GA but not poly-PR in mice triggers selective neuron loss and interferon responses found in C9orf72 ALS. *Acta Neuropathol* **140**, 121-142 (2020).
102 Hao, Z. *et al.* Motor dysfunction and neurodegeneration in a C9orf72 mouse line expressing poly-PR. *Nat Commun* **10**, 2906 (2019).
103 Choi, S. Y. *et al.* C9ORF72-ALS/FTD-associated poly(GR) binds Atp5a1 and compromises mitochondrial function in vivo. *Nat Neurosci* **22**, 851-862 (2019).
104 Zhang, Y. J. *et al.* Poly(GR) impairs protein translation and stress granule dynamics in C9orf72-associated frontotemporal dementia and amyotrophic lateral sclerosis. *Nat Med* **24**, 1136-1142 (2018).
105 Koppers, M. *et al.* C9orf72 ablation in mice does not cause motor neuron degeneration or motor deficits. *Ann Neurol* **78**, 426-438 (2015).
106 Burberry, A. *et al.* Loss-of-function mutations in the C9ORF72 mouse ortholog cause fatal autoimmune disease. *Sci Transl Med* **8**, 347ra393 (2016).
107 Lagier-Tourenne, C. *et al.* Targeted degradation of sense and antisense C9orf72 RNA foci as therapy for ALS and frontotemporal degeneration. *Proc Natl Acad Sci U S A* **110**, E4530-4539 (2013).
108 Yu, X., Nagai, J. & Khakh, B. S. Improved tools to study astrocytes. *Nat Rev Neurosci* **21**, 121-138 (2020).

109 Rostalski, H. *et al.* Astrocytes and Microglia as Potential Contributors to the Pathogenesis of C9orf72 Repeat Expansion-Associated FTLD and ALS. *Front Neurosci* **13**, 486 (2019).
110 Yamanaka, K. *et al.* Astrocytes as determinants of disease progression in inherited amyotrophic lateral sclerosis. *Nat Neurosci* **11**, 251-253 (2008).
111 Gong, Y. H., Parsadanian, A. S., Andreeva, A., Snider, W. D. & Elliott, J. L. Restricted Expression of G86R Cu/Zn Superoxide Dismutase in Astrocytes Results in Astrocytosis But Does Not Cause Motoneuron Degeneration. *The Journal of Neuroscience* **20**, 660-665 (2000).
112 Wang, L., Gutmann, D. H. & Roos, R. P. Astrocyte loss of mutant SOD1 delays ALS disease onset and progression in G85R transgenic mice. *Hum Mol Genet* **20**, 286-293 (2011).
113 Lepore, A. C. *et al.* Focal transplantation-based astrocyte replacement is neuroprotective in a model of motor neuron disease. *Nat Neurosci* **11**, 1294-1301 (2008).
114 Papadeas, S. T., Kraig, S. E., O'Banion, C., Lepore, A. C. & Maragakis, N. J. Astrocytes carrying the superoxide dismutase 1 (SOD1G93A) mutation induce wild-type motor neuron degeneration in vivo. *Proc Natl Acad Sci U S A* **108**, 17803-17808 (2011).
115 Nagai, M. *et al.* Astrocytes expressing ALS-linked mutated SOD1 release factors selectively toxic to motor neurons. *Nat Neurosci* **10**, 615-622 (2007).
116 Di Giorgio, F. P., Carrasco, M. A., Siao, M. C., Maniatis, T. & Eggan, K. Non-cell autonomous effect of glia on motor neurons in an embryonic stem cell-based ALS model. *Nat Neurosci* **10**, 608-614 (2007).
117 Di Giorgio, F. P., Boulting, G. L., Bobrowicz, S. & Eggan, K. C. Human embryonic stem cell-derived motor neurons are sensitive to the toxic effect of glial cells carrying an ALS-causing mutation. *Cell Stem Cell* **3**, 637-648 (2008).
118 Marchetto, M. C. *et al.* Non-cell-autonomous effect of human SOD1 G37R astrocytes on motor neurons derived from human embryonic stem cells. *Cell Stem Cell* **3**, 649-657 (2008).
119 Haidet-Phillips, A. M. *et al.* Astrocytes from familial and sporadic ALS patients are toxic to motor neurons. *Nat Biotechnol* **29**, 824-828 (2011).
120 Phatnani, H. P. *et al.* Intricate interplay between astrocytes and motor neurons in ALS. *Proc Natl Acad Sci U S A* **110**, E756-765 (2013).
121 Ferraiuolo, L. *et al.* Dysregulation of astrocyte-motoneuron cross-talk in mutant superoxide dismutase 1-related amyotrophic lateral sclerosis. *Brain* **134**, 2627-2641 (2011).
122 Vargas, M. R., Johnson, D. A., Sirkis, D. W., Messing, A. & Johnson, J. A. Nrf2 activation in astrocytes protects against neurodegeneration in mouse models of familial amyotrophic lateral sclerosis. *J Neurosci* **28**, 13574-13581 (2008).
123 Pehar, M., Harlan, B. A., Killoy, K. M. & Vargas, M. R. Role and Therapeutic Potential of Astrocytes in Amyotrophic Lateral Sclerosis. *Curr Pharm Des* **23**, 5010-5021 (2017).
124 Yamanaka, K. & Komine, O. The multi-dimensional roles of astrocytes in ALS. *Neurosci Res* **126**, 31-38 (2018).
125 Endo, F. *et al.* Astrocyte-derived TGF-beta1 accelerates disease progression in ALS mice by interfering with the neuroprotective functions of microglia and T cells. *Cell Rep* **11**, 592-604 (2015).
126 Guttenplan, K. A. *et al.* Knockout of reactive astrocyte activating factors slows disease progression in an ALS mouse model. *Nat Commun* **11**, 3753 (2020).
127 Mishra, V. *et al.* Systematic elucidation of neuron-astrocyte interaction in models of amyotrophic lateral sclerosis using multi-modal integrated bioinformatics workflow. *Nat Commun* **11**, 5579 (2020).

128 Howland, D. S. *et al.* Focal loss of the glutamate transporter EAAT2 in a transgenic rat model of SOD1 mutant-mediated amyotrophic lateral sclerosis (ALS). *Proc Natl Acad Sci U S A* **99**, 1604-1609 (2002).
129 Bruijn, L. I. *et al.* ALS-linked SOD1 mutant G85R mediates damage to astrocytes and promotes rapidly progressive disease with SOD1-containing inclusions. *Neuron* **18**, 327-338 (1997).
130 Ditsworth, D. *et al.* Mutant TDP-43 within motor neurons drives disease onset but not progression in amyotrophic lateral sclerosis. *Acta Neuropathol* **133**, 907-922 (2017).
131 Tong, J. *et al.* Expression of ALS-linked TDP-43 mutant in astrocytes causes non-cell-autonomous motor neuron death in rats. *EMBO J* **32**, 1917-1926 (2013).
132 Velebit, J. *et al.* Astrocytes with TDP-43 inclusions exhibit reduced noradrenergic cAMP and Ca(2+) signaling and dysregulated cell metabolism. *Sci Rep* **10**, 6003 (2020).
133 Moujalled, D. *et al.* TDP-43 mutations causing amyotrophic lateral sclerosis are associated with altered expression of RNA-binding protein hnRNP K and affect the Nrf2 antioxidant pathway. *Hum Mol Genet* **26**, 1732-1746 (2017).
134 Rojas, F., Cortes, N., Abarzua, S., Dyrda, A. & van Zundert, B. Astrocytes expressing mutant SOD1 and TDP43 trigger motoneuron death that is mediated via sodium channels and nitroxidative stress. *Front Cell Neurosci* **8**, 24 (2014).
135 Huang, C. *et al.* Profiling the genes affected by pathogenic TDP-43 in astrocytes. *J Neurochem* **129**, 932-939 (2014).
136 Bi, F. *et al.* Reactive astrocytes secrete lcn2 to promote neuron death. *Proc Natl Acad Sci U S A* **110**, 4069-4074 (2013).
137 Iguchi, Y. *et al.* Loss of TDP-43 causes age-dependent progressive motor neuron degeneration. *Brain* **136**, 1371-1382 (2013).
138 Wu, L. S., Cheng, W. C. & Shen, C. K. Targeted depletion of TDP-43 expression in the spinal cord motor neurons leads to the development of amyotrophic lateral sclerosis-like phenotypes in mice. *J Biol Chem* **287**, 27335-27344 (2012).
139 Yang, C. *et al.* Partial loss of TDP-43 function causes phenotypes of amyotrophic lateral sclerosis. *Proc Natl Acad Sci U S A* **111**, E1121-1129 (2014).
140 LaRocca, T. J., Mariani, A., Watkins, L. R. & Link, C. D. TDP-43 knockdown causes innate immune activation via protein kinase R in astrocytes. *Neurobiol Dis* **132**, 104514 (2019).
141 Kraemer, B. C. *et al.* Loss of murine TDP-43 disrupts motor function and plays an essential role in embryogenesis. *Acta Neuropathol* **119**, 409-419 (2010).
142 Chiang, P. M. *et al.* Deletion of TDP-43 down-regulates Tbc1d1, a gene linked to obesity, and alters body fat metabolism. *Proc Natl Acad Sci U S A* **107**, 16320-16324 (2010).
143 Serio, A. *et al.* Astrocyte pathology and the absence of non-cell autonomy in an induced pluripotent stem cell model of TDP-43 proteinopathy. *Proc Natl Acad Sci U S A* **110**, 4697-4702 (2013).
144 Haidet-Phillips, A. M. *et al.* Altered astrocytic expression of TDP-43 does not influence motor neuron survival. *Exp Neurol* **250**, 250-259 (2013).
145 Smethurst, P. *et al.* Distinct responses of neurons and astrocytes to TDP-43 proteinopathy in amyotrophic lateral sclerosis. *Brain* **143**, 430-440 (2020).
146 Chew, J. *et al.* Neurodegeneration. C9ORF72 repeat expansions in mice cause TDP-43 pathology, neuronal loss, and behavioral deficits. *Science* **348**, 1151-1154 (2015).
147 Zhang, Y. J. *et al.* C9ORF72 poly(GA) aggregates sequester and impair HR23 and nucleocytoplasmic transport proteins. *Nat Neurosci* **19**, 668-677 (2016).
148 Chew, J. *et al.* Aberrant deposition of stress granule-resident proteins linked to C9orf72-associated TDP-43 proteinopathy. *Mol Neurodegener* **14**, 9 (2019).

149 Meyer, K. *et al.* Direct conversion of patient fibroblasts demonstrates non-cell autonomous toxicity of astrocytes to motor neurons in familial and sporadic ALS. *Proc Natl Acad Sci U S A* **111**, 829-832 (2014).
150 Hautbergue, G. M. *et al.* SRSF1-dependent nuclear export inhibition of C9ORF72 repeat transcripts prevents neurodegeneration and associated motor deficits. *Nat Commun* **8**, 16063 (2017).
151 Birger, A. *et al.* Human iPSC-derived astrocytes from ALS patients with mutated C9ORF72 show increased oxidative stress and neurotoxicity. *EBioMedicine* **50**, 274-289 (2019).
152 Allen, S. P. *et al.* Astrocyte adenosine deaminase loss increases motor neuron toxicity in amyotrophic lateral sclerosis. *Brain* **142**, 586-605 (2019).
153 Allen, S. P. *et al.* C9orf72 expansion within astrocytes reduces metabolic flexibility in amyotrophic lateral sclerosis. *Brain* **142**, 3771-3790 (2019).
154 Fomin, V. *et al.* The C9ORF72 Gene, Implicated in Amyotrophic Lateral Sclerosis and Frontotemporal Dementia, Encodes a Protein That Functions in Control of Endothelin and Glutamate Signaling. *Mol Cell Biol* **38**, e00155-00118 (2018).
155 Varcianna, A. *et al.* Micro-RNAs secreted through astrocyte-derived extracellular vesicles cause neuronal network degeneration in C9orf72 ALS. *EBioMedicine* **40**, 626-635 (2019).
156 Thompson, A. G. *et al.* Extracellular vesicles in neurodegenerative disease - pathogenesis to biomarkers. *Nat Rev Neurol* **12**, 346-357 (2016).
157 Zhao, C. *et al.* Mutant C9orf72 human iPSC-derived astrocytes cause non-cell autonomous motor neuron pathophysiology. *Glia* **68**, 1046-1064 (2020).
158 Kang, S. H. *et al.* Degeneration and impaired regeneration of gray matter oligodendrocytes in amyotrophic lateral sclerosis. *Nat Neurosci* **16**, 571-579 (2013).
159 Philips, T. *et al.* Oligodendrocyte dysfunction in the pathogenesis of amyotrophic lateral sclerosis. *Brain* **136**, 471-482 (2013).
160 Yamanaka, K. *et al.* Mutant SOD1 in cell types other than motor neurons and oligodendrocytes accelerates onset of disease in ALS mice. *Proc Natl Acad Sci U S A* **105**, 7594-7599 (2008).
161 Ferraiuolo, L. *et al.* Oligodendrocytes contribute to motor neuron death in ALS via SOD1-dependent mechanism. *Proc Natl Acad Sci U S A* **113**, E6496-E6505 (2016).
162 Lee, Y. *et al.* Oligodendroglia metabolically support axons and contribute to neurodegeneration. *Nature* **487**, 443-448 (2012).
163 Kim, S. *et al.* Myelin degeneration induced by mutant superoxide dismutase 1 accumulation promotes amyotrophic lateral sclerosis. *Glia* **67**, 1910-1921 (2019).
164 Sun, S. *et al.* Translational profiling identifies a cascade of damage initiated in motor neurons and spreading to glia in mutant SOD1-mediated ALS. *Proc Natl Acad Sci U S A* **112**, E6993-7002 (2015).
165 Livesey, M. R. *et al.* Maturation and electrophysiological properties of human pluripotent stem cell-derived oligodendrocytes. *Stem Cells* **34**, 1040-1053 (2016).
166 Butovsky, O. *et al.* Modulating inflammatory monocytes with a unique microRNA gene signature ameliorates murine ALS. *J Clin Invest* **122**, 3063-3087 (2012).
167 Zhao, W. *et al.* Characterization of Gene Expression Phenotype in Amyotrophic Lateral Sclerosis Monocytes. *JAMA Neurol* **74**, 677-685 (2017).
168 Du, Y. *et al.* Increased activation ability of monocytes from ALS patients. *Exp Neurol* **328**, 113259 (2020).
169 Zondler, L. *et al.* Peripheral monocytes are functionally altered and invade the CNS in ALS patients. *Acta Neuropathol* **132**, 391-411 (2016).
170 McCauley, M. E. *et al.* C9orf72 in myeloid cells suppresses STING-induced inflammation. *Nature* **585**, 96-101 (2020).

171 Park, L. *et al.* Brain Perivascular Macrophages Initiate the Neurovascular Dysfunction of Alzheimer Abeta Peptides. *Circ Res* **121**, 258-269 (2017).
172 Chiu, I. M. *et al.* T lymphocytes potentiate endogenous neuroprotective inflammation in a mouse model of ALS. *Proc Natl Acad Sci U S A* **105**, 17913-17918 (2008).
173 Beers, D. R., Henkel, J. S., Zhao, W., Wang, J. & Appel, S. H. CD4+ T cells support glial neuroprotection, slow disease progression, and modify glial morphology in an animal model of inherited ALS. *Proc Natl Acad Sci U S A* **105**, 15558-15563 (2008).
174 Beers, D. R. *et al.* Endogenous regulatory T lymphocytes ameliorate amyotrophic lateral sclerosis in mice and correlate with disease progression in patients with amyotrophic lateral sclerosis. *Brain* **134**, 1293-1314 (2011).
175 Zhao, W., Beers, D. R., Liao, B., Henkel, J. S. & Appel, S. H. Regulatory T lymphocytes from ALS mice suppress microglia and effector T lymphocytes through different cytokine-mediated mechanisms. *Neurobiol Dis* **48**, 418-428 (2012).
176 Beers, D. R. *et al.* ALS patients' regulatory T lymphocytes are dysfunctional, and correlate with disease progression rate and severity. *JCI Insight* **2**, e89530 (2017).
177 Henkel, J. S. *et al.* Regulatory T-lymphocytes mediate amyotrophic lateral sclerosis progression and survival. *EMBO Mol Med* **5**, 64-79 (2013).
178 Sheean, R. K. *et al.* Association of Regulatory T-Cell Expansion With Progression of Amyotrophic Lateral Sclerosis: A Study of Humans and a Transgenic Mouse Model. *JAMA Neurol* **75**, 681-689 (2018).
179 Komine, O. *et al.* Innate immune adaptor TRIF deficiency accelerates disease progression of ALS mice with accumulation of aberrantly activated astrocytes. *Cell Death Differ* **25**, 2130-2146 (2018).
180 Nardo, G. *et al.* Counteracting roles of MHCI and CD8(+) T cells in the peripheral and central nervous system of ALS SOD1(G93A) mice. *Mol Neurodegener* **13**, 42 (2018).
181 Coque, E. *et al.* Cytotoxic CD8(+) T lymphocytes expressing ALS-causing SOD1 mutant selectively trigger death of spinal motoneurons. *Proc Natl Acad Sci U S A* **116**, 2312-2317 (2019).
182 Atanasio, A. *et al.* C9orf72 ablation causes immune dysregulation characterized by leukocyte expansion, autoantibody production, and glomerulonephropathy in mice. *Sci Rep* **6**, 23204 (2016).
183 Zhao, W. *et al.* Immunosuppressive Functions of M2 Macrophages Derived from iPSCs of Patients with ALS and Healthy Controls. *iScience* **23**, 101192 (2020).
184 Finkelstein, A. *et al.* Abnormal changes in NKT cells, the IGF-1 axis, and liver pathology in an animal model of ALS. *PLoS One* **6**, e22374 (2011).
185 Murdock, B. J. *et al.* Correlation of Peripheral Immunity With Rapid Amyotrophic Lateral Sclerosis Progression. *JAMA Neurol* **74**, 1446-1454 (2017).
186 Vijayakumar, U. G. *et al.* A Systematic Review of Suggested Molecular Strata, Biomarkers and Their Tissue Sources in ALS. *Front Neurol* **10**, 400 (2019).
187 Verber, N. & Shaw, P. J. Biomarkers in amyotrophic lateral sclerosis: a review of new developments. *Curr Opin Neurol* **33**, 662-668 (2020).
188 Varghese, A. M. *et al.* Chitotriosidase - a putative biomarker for sporadic amyotrophic lateral sclerosis. *Clin Proteomics* **10**, 19 (2013).
189 Steinacker, P. *et al.* Chitotriosidase (CHIT1) is increased in microglia and macrophages in spinal cord of amyotrophic lateral sclerosis and cerebrospinal fluid levels correlate with disease severity and progression. *J Neurol Neurosurg Psychiatry* **89**, 239-247 (2018).
190 Varghese, A. M. *et al.* Chitotriosidase, a biomarker of amyotrophic lateral sclerosis, accentuates neurodegeneration in spinal motor neurons through neuroinflammation. *J Neuroinflammation* **17**, 232 (2020).

191 Thompson, A. G. *et al.* CSF chitinase proteins in amyotrophic lateral sclerosis. *J Neurol Neurosurg Psychiatry* **90**, 1215-1220 (2019).
192 Gray, E. *et al.* CSF chitinases before and after symptom onset in amyotrophic lateral sclerosis. *Ann Clin Transl Neurol* (2020).
193 Oeckl, P. *et al.* Different neuroinflammatory profile in amyotrophic lateral sclerosis and frontotemporal dementia is linked to the clinical phase. *J Neurol Neurosurg Psychiatry* **90**, 4-10 (2019).
194 Gille, B. *et al.* Inflammatory markers in cerebrospinal fluid: independent prognostic biomarkers in amyotrophic lateral sclerosis? *J Neurol Neurosurg Psychiatry* **90**, 1338-1346 (2019).
195 Illan-Gala, I. *et al.* CSF sAPPbeta, YKL-40, and NfL along the ALS-FTD spectrum. *Neurology* **91**, e1619-e1628 (2018).
196 Abu-Rumeileh, S. *et al.* Diagnostic-prognostic value and electrophysiological correlates of CSF biomarkers of neurodegeneration and neuroinflammation in amyotrophic lateral sclerosis. *J Neurol* **267**, 1699-1708 (2020).
197 Gaur, N., Perner, C., Witte, O. W. & Grosskreutz, J. The Chitinases as Biomarkers for Amyotrophic Lateral Sclerosis: Signals From the CNS and Beyond. *Front Neurol* **11**, 377 (2020).
198 Castellani, R. J., Perry, G. & Smith, M. A. The role of novel chitin-like polysaccharides in Alzheimer disease. *Neurotox Res* **12**, 269-274 (2007).
199 Vu, L. *et al.* Cross-sectional and longitudinal measures of chitinase proteins in amyotrophic lateral sclerosis and expression of CHI3L1 in activated astrocytes. *J Neurol Neurosurg Psychiatry* **91**, 350-358 (2020).
200 Andres-Benito, P. *et al.* YKL40 in sporadic amyotrophic lateral sclerosis: cerebrospinal fluid levels as a prognosis marker of disease progression. *Aging (Albany NY)* **10**, 2367-2382 (2018).
201 Chen, Y., Xia, K., Chen, L. & Fan, D. Increased Interleukin-6 Levels in the Astrocyte-Derived Exosomes of Sporadic Amyotrophic Lateral Sclerosis Patients. *Front Neurosci* **13**, 574 (2019).
202 Henkel, J. S., Beers, D. R., Siklos, L. & Appel, S. H. The chemokine MCP-1 and the dendritic and myeloid cells it attracts are increased in the mSOD1 mouse model of ALS. *Mol Cell Neurosci* **31**, 427-437 (2006).
203 Huang, F. *et al.* Longitudinal biomarkers in amyotrophic lateral sclerosis. *Ann Clin Transl Neurol* **7**, 1103-1116 (2020).
204 Kuhle, J. *et al.* Increased levels of inflammatory chemokines in amyotrophic lateral sclerosis. *Eur J Neurol* **16**, 771-774 (2009).
205 Guo, J., Yang, X., Gao, L. & Zang, D. Evaluating the levels of CSF and serum factors in ALS. *Brain Behav* **7**, e00637 (2017).
206 Lehnert, S. *et al.* Multicentre quality control evaluation of different biomarker candidates for amyotrophic lateral sclerosis. *Amyotroph Lateral Scler Frontotemporal Degener* **15**, 344-350 (2014).
207 Nagata, T. *et al.* Elevation of MCP-1 and MCP-1/VEGF ratio in cerebrospinal fluid of amyotrophic lateral sclerosis patients. *Neurol Res* **29**, 772-776 (2007).
208 Wilms, H. *et al.* Intrathecal synthesis of monocyte chemoattractant protein-1 (MCP-1) in amyotrophic lateral sclerosis: further evidence for microglial activation in neurodegeneration. *J Neuroimmunol* **144**, 139-142 (2003).
209 Kiernan, M. C. *et al.* Improving clinical trial outcomes in amyotrophic lateral sclerosis. *Nat Rev Neurol* (2020).
210 Mora, J. S. *et al.* Masitinib as an add-on therapy to riluzole in patients with amyotrophic lateral sclerosis: a randomized clinical trial. *Amyotroph Lateral Scler Frontotemporal Degener* **21**, 5-14 (2020).

1290

1291

Acknowledgements

BFV is funded by the University of Oxford Clarendon Fund, St John's College Oxford, the Oxford-Medical Research Council (MRC) Doctoral Training Partnership, and the National Institute for Health Research (NIHR) Oxford Biomedical Research Centre.

EG is supported by the Motor Neurone Disease Association.

AGT is supported by the Medical Research Council & Motor Neurone Disease Association Lady Edith Wolfson Clinician Scientist Fellowship.

SAC is supported through the Oxford Martin School.

MRT is supported by the Motor Neurone Disease Association.

The views expressed are those of the author(s) and not necessarily those of the National Health Service, the NIHR or the Department of Health and Social Care.

Author contributions

BFV drafted the manuscript.

EG, AGT, OA, DCA, SAC, KT & MRT edited the manuscript.

Competing interests

The authors declare no competing interests.

Key points

- An exclusively motor neuron-centred model of pathogenesis in ALS is untenable, and this has important implications for therapeutic development strategies
- Resident microglia, astrocytes, oligodendrocytes, but also peripheral immune cells, all exert highly relevant functions in CNS homeostasis and physiology
- In multiple experimental models, non-neuronal cells may exert neurotoxic effects via both gain- and loss-of-function mechanisms, but also show apparently neuroprotective activity
- Many of the hypotheses around non-neuronal cells in ALS pathogenesis were developed using rodent models, some with limited relevance to the TDP-43 neuropathological hallmark of human ALS
- Human induced pluripotent stem cells permit the investigation of non-neuronal cells carrying ALS-associated genetic mutations, but multi-cellular co-cultures may be needed to disentangle their nuanced interactions with motor neurons
- Human biofluid biomarkers derived from non-neuronal cells offer an important window into understanding the *in vivo* pathological milieu, with potential as earlier markers of therapeutic response

Display items

Tables

Table 1: Techniques to study non-neuronal cell involvement in ALS

Technique	Main features	Advantages	Limitations
Neuropathology	Morphology, immunostaining, molecular phenotyping including spatial transcriptomics and proteomics	High resolution characterization of human disease-specific tissue, retaining cell relationships	Static, descriptive and enriched for phenomena associated with end stage processes
Human CNS imaging	MRI (magnetic resonance imaging), PET (positron emission tomography)	MRI: in vivo analysis of structure and some aspects of function. Allows longitudinal changes including at pre-symptomatic stages. PET: (relatively) cell-specific tracers can assess activation of specific non-neuronal cells	Low resolution does not allow analysis of individual cells
Rodent models	Mouse and rat transgenic models expressing human cDNA or genomic constructs with specific genetic mutations; primary cell lines	Mammalian system with retention of complex multicellular environment; genetically manipulatable to allow cell-specific expression; allow spatial and temporal analysis of pathophysiology	Species differences (immune, microbiome, divergence in protein functions) between human and rodent may be a barrier to translation; phenotypes may depend on no-physiological overexpression; modelling age-related neurodegeneration is challenging
Human primary cell lines	Fibroblasts or induced pluripotent stem cell derived glia	Allow analysis of human cells on genetic background including disease-causing mutations. Avoid non-physiological overexpression and inter-species differences in the transcriptome. Useful to study cell-autonomous and non-cell-autonomous effects	Cells may be arrested in early developmental stages; expensive and labour intensive; considerable inter-individual variance requires isogenic controls
Human induced Pluripotent Stem Cell models	Complex multicellular cultures derived from iPSCs	Multiple cell-cell interactions; experimental control of microenvironment	Relationship to normal tissue architecture imperfect. May favour developmental studies

Figure legends

Figure 1: **Overview of the pathophysiological role of microglia, astrocytes, and oligodendrocytes in ALS.** Neuronal damage associated with protein dysfunction and aggregation (SOD1, TDP-43, DPRs) as well as aggregate release are associated with the activation of glial cells (yellow arrows). The expression of ALS-associated genetic mutations (*SOD1*, *TDP-43*, *C9orf72*) has been linked with intrinsic neurotoxic properties in non-neuronal cells, both through gain-of-function toxicity (red arrows) and loss of supportive functions (green dashed arrows), exacerbated by crosstalk between different cell-types. The mechanisms depicted give a combined overview on data obtained in the different models discussed in this review, but not all of them have been identified in all models. Figure created with BioRender.com

Figure 2: **Overview of the pathophysiological role of peripheral non-neuronal cells in ALS.** Neuronal damage associated with protein dysfunction and aggregation (SOD1, TDP-43, DPRs) is associated with the recruitment of peripheral non-neuronal cells, where chemotactic factors such as MCP-1/CCL2 are likely to play a role (yellow arrows). The expression of ALS-associated genetic mutations (*SOD1*, *TDP-43*, *C9orf72*) has been linked with intrinsic neurotoxic properties in non-neuronal cells both through gain-of-function toxicity (red arrows) and loss of supportive functions (green dashed arrows), exacerbated by crosstalk between different cell-types. The mechanisms depicted give a combined overview on data obtained in the different models discussed in this review, but not all of them have been identified in all models. Figure created with BioRender.com