Immune dysregulation in amyotrophic lateral sclerosis: mechanisms and emerging therapies

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Neuroinflammation is a common pathological feature of many neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS), and is characterised by activated CNS microglia and astroglia, proinflammatory peripheral lymphocytes, and macrophages. Data from clinical studies show that multiple genetic mutations linked to ALS (eg, mutations in *SOD1*, *TARDBP*, and *C9orf72*) enhance this neuroinflammation, which provides compelling evidence for immune dysregulation in the pathogenesis of ALS. Transgenic rodent models expressing these mutations induce an ALS-like disease with accompanying inflammatory responses, confirming the immune system's involvement in disease progression. Even in the absence of known genetic alterations, immune dysregulation has been shown to lead to dysfunctional regulatory T lymphocytes and increased proinflammatory macrophages in clinical studies. Therefore, an improved understanding of the biological processes that induce this immune dysregulation will help to identify therapeutic strategies that circumvent or ameliorate the pathogenesis of ALS. Emerging cell-based therapies hold the promise of accomplishing this goal and, therefore, improving quality of life and extending survival in patients with ALS.

Lancet Neurol 2019; 18: 211–20

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Introduction

The clinical presentation of amyotrophic lateral sclerosis (ALS) is heterogeneous regarding age and site of disease onset, rate of disease progression, and survival. Evidence from clinical studies suggests that a dysregulated immune response contributes to this heterogeneity.[1] Although ALS is not initiated by immune alterations, disease progression is amplified by activated CNS microglia and inflammatory reactions of peripheral lymphocytes and macrophages (panel).[2] However, studies in patients with ALS and transgenic mice have shown that these inflammatory reactions are dual in nature—ie, an initial protective response (anti-inflammatory) is followed by a subsequent toxic response (proinflammatory).[56,83,84] These early versus late inflammatory responses have been similarly associated with other neurodegenerative diseases, such as Parkinson's disease and Alzheimer's disease, and represent potential therapeutic targets.[11,12]

The pathophysiological processes underlying ALS are multifactorial and reflect a complex interaction between genetic and environmental factors.[13] Clinical studies have shown that mutations in genes such as superoxide dismutase 1 (*SOD1*), TAR DNA-binding protein 43 (*TARDBP*), and chromosome 9 open reading frame 72 (*C9orf72*) impair degradation of aggregated proteins, compromise CNS glial protective responses, and promote proinflammatory-mediated motoneuron injury.[13–22] These genetic mutations have also been studied in transgenic rodent models and have provided evidence that diverse inflammatory responses could contribute to progression of disease, motoneuron dysfunction, and death. Regardless of which genetic mutation initiates motoneuron injury in patients with ALS, the pathogenic process might be similar—ie, multiple molecular mechanisms can converge leading to an inflammatory cascade. Therefore, diverse gene mutations can result in the same clinical phenotype and different clinical phenotypes can result from the same mutant gene.[18] Motoneuron viability and death are the culmination of both motoneuron autonomous and non-cell autonomous processes mediated by CNS glia and peripheral innate and adaptive immune responses.

This Review aims to elucidate the role of neuroinflammation in the pathology of ALS. We begin with a presentation of the ALS-linked mutant immune genes that provide evidence that immune dysregulation can both initiate and contribute to the pathogenesis of ALS, followed by a discussion of the transgenic rodent models of ALS that help to define the potential mechanisms involved in the pathogenesis. Even in the absence of a positive family history of ALS, immune dysfunction results in increased central and peripheral inflammatory responses. Previous efforts to suppress these inflammatory responses have largely been ineffective because of the dual nature (ie, early *vs* late response) of these responses, but emerging cell-based therapies hold the promise of ameliorating the immune dysregulation, thus improving quality of life and survival of patients with ALS.

Immune-related genes linked to ALS

Advances in gene sequencing have led to the discovery of several mutant genes that cause ALS. Many of the proteins encoded by these mutant genes compromise immune system function and provide important evidence that immune dysregulation contributes to the pathogenesis of ALS. Marked inflammation within the CNS is present in patients with ALS due to mutations in *SOD1*, *TARDBP*, or *C9orf72*.[2,19–22] Transgenic rodent models show that expression of these mutant genes leads to an ALS-like disease and promotes immune dysfunction. Mutations in *OPTN* (coding for optineurin [OPTN] protein), *TBK1* (coding for serine–threonine protein kinase-binding kinase 1), *SQSTM1* (coding for sequestosome-1 protein, also known as ubiquitin-binding protein p62), *TNIP1* (coding for tumour necrosis factor α-induced protein 3 interacting protein 1), *VPC* (coding for valosin-containing protein), and *CX3CR1* (coding for chemokine receptor 1,

Panel: Glossary of terms

Astrocytes

Astrocytes are the most abundant glia cell type of the CNS and are active players in neuroinflammation; their response might be beneficial or detrimental for tissue repair, depending on environmental signalling.[3] In vivo studies have provided compelling evidence that astrocyte responses to certain cytokines, growth factors, and hormones are protective, whereas their absence worsens CNS injury.[4] The major protective pathway is mediated by the glycoprotein gp130, an essential signal transducer for members of the interleukin (IL)-6 cytokine family. Tumour growth factor (TGF)-β is released from astrocytes because of its important immunosuppressive properties (ie, suppresses the neuroprotective function of microglia and T cells, resulting in shortened lifespan), and is produced by microglia cells and astrocytes under healthy conditions and strongly upregulated after CNS injury. The TGF-β pathway is upregulated in astrocytes in rodent models and patients with amyotropphic lateral sclerosis (ALS), as a negative regulator of the neuroprotective inflammatory response, and is a crucial mediator of the pathomechanism of ALS.[4]

Macrophages

Macrophages originate from blood monocytes (large circulating white blood cells formed in the bone marrow) and migrate to tissues where they serve diverse immune functions, switching from one functional phenotype to another by secreting proinflammatory or anti-inflammatory cytokines. Macrophages participate in the inflammatory cascade and its eventual resolution.[5] Macrophage activation is dynamic, plastic, rapid, and fully reversible.[5] The concept of macrophage activation varies along a continuum, from a proinflammatory (M1 macrophages) to an anti-inflammatory phenotype (M2 macrophages). The process of changing the macrophage phenotype is called reprogramming (also known as polarisation or alternate phenotype). However, to date, no pure macrophage phenotype having only M1 or M2 markers has been described. Therefore, it is considered that the M1 phenotype has more M1 than M2 markers and vice versa. Furthermore, it has been proposed that factors which shift macrophage phenotype towards M1 (such as interferon-γ) should be referred to as reprogramming factor-M1, and those which shift the phenotype towards M2 (such as IL-4)[5] as reprogramming factor-M2.

Microglia

Microglia are the resident parenchymal myeloid cells of the CNS, with important roles in development, homoeostasis, and injury.[2] They originate from primitive yolk sac macrophages and, after microglial populations are established within the CNS, they are maintained throughout life by local proliferation and are not replaced by bone-marrow-derived peripheral circulating cells. Microglia are one of the first lines of defence for the CNS against injury and infection.[6] Microglia, in addition to sampling the environment and their roles as antigen-presenting cells, have pattern recognition receptors, such as cluster differentiation 14 and toll-like receptors 2 and 4, which initiate an innate immune response and induce motor neuron death via the classical nuclear factor-κappa B pathway in ALS (figure 1).[7] Microglia promote cytotoxicity by secreting reactive oxygen species and proinflammatory cytokines, including IL-1, IL-6, and tumour necrosis factor-α, and a reduction in protective trophic factors. However, microglia can also produce high levels of anti-inflammatory cytokines and neurotrophic factors including IL-4, IL-10, and insulin-like growth factor-1.[7]

also known as fractalkine receptor) have also been reported to directly compromise immune gene function and promote inflammatory responses (table). These mutant genes are the most direct evidence that CNS and peripheral immune system driven inflammatory mechanisms are involved in the pathogenesis of ALS. They are also an indication that autophagy (ie, the intracellular process that allows the sequestering and orderly degradation and recycling of aggregated misfolded proteins and dysfunctional cellular organelles), nuclear factor-kappa B (NF-κB), and the downstream nucleotide-binding domain-like receptor protein 3 (NLRP3) inflammasome (ie, a multiprotein complex responsible for the activation of inflammatory responses that promotes the maturation and secretion of proinflammatory cytokines interleukin [IL]-1β and IL-18) contribute to the pathogenesis of ALS.

OPTN is the only known gene that is thought to cause classic ALS by a loss-of-function mutation.[31] *OPTN* normally suppresses NF-κB activity, a key component of the innate immune response and, in its absence or mutant form, NF-κB is translocated to the nucleus and promotes expression of a large number of proinflammatory genes enhancing microglial-mediated neuroinflammation (figure 1).[14,15,31] Whether *OPTN* directly affects NF-κB is a controversial topic; however, most studies agree that mutant *OPTN* is associated with dysregulation of the NF-κB pathway, thereby promoting a proinflammatory response.[15,32]

Mutations in *TBK1* are linked with ALS. The TBK1 protein binds to and phosphorylates a number of proteins, including OPTN and sequestosome-1/p62, and regulates both innate immunity and autophagy.[16,38] Mutations in *TBK1* lead to both ALS and frontotemporal dementia.[16] Cosegregation (ie, the transmission of two or more linked genes on a chromosome leading to inheritance of these genes together) and haploinsufficiency (ie, when one copy of a gene is inactivated or deleted and the remaining functional copy of the gene is not adequate to produce the needed gene product to preserve normal function) of *TBK1* mutations also result in ALS and frontotemporal dementia.[39,40]

Mutations in *TNIP1* have been associated with ALS on the basis of a large genome-wide association study in Chinese, European, and Australian populations.[43] TNIP1

is functionally associated with OPTN and inhibits NF-κB activation and tumour necrosis factor (TNF)-induced NF-κB-dependent gene expression. *TNIP1* has also been linked to several immunological diseases, including lupus and psoriasis.[44,47]

Several novel *SQSTM1* mutations have been identified in patients with ALS.[37] The *SQSTM1* gene encodes p62, a major pathological protein that regulates autophagy and oxidative stress (figure 2).[36,37] The putative mechanism of action, investigated in double transgenic *SQSTM1* and *SOD1* mice, is impaired protein degradation based on the presence of insoluble SQSTM1, as well as other poly-ubiquinated proteins.[36] Mutations in *SQSTM1* alter the function of p62 and contribute to the pathophysiology of ALS by impairing both aggregated protein degradation and autophagy.[36,37]

Mutations in the *VCP* gene have been linked to both familial and sporadic ALS.[45,48] VCP is an essential component of both the autophagy and the ubiquitination-proteasomal pathways (another cellular mechanism of degrading and disposing of damaged, misfolded, and excess of proteins). Mutations in *VCP* impair overall protein degradation and lead to TDP-43 deposition, resulting in inclusion body myopathy, Paget's disease, frontotemporal dementia, or ALS.[45]

CX3CR1 is a specific receptor on microglia that binds to fractalkine, a protein released from motoneurons, which thereby promots a neuroprotective response. Mutations in the receptor CX3CR1 impair fractalkine binding and lead to shortened survival time in patients wth ALS but do not increase the risk of disease.[29,30] Nevertheless, *CX3CR1* is an ALS disease-modifying gene; polymorphisms of *CX3CR1* can impair the neuroprotective responses of innate immune microglia,[29,30] providing evidence for its role on neuroinflammation in ALS disease pathogenesis.

These mutant genes provide direct evidence that immune system-induced inflammatory mechanisms are involved in the pathogenesis of ALS. Furthermore, these mutant genes indicate that autophagy inhibits the activation of the NLRP3 inflammasome, and mutations in these immune-related genes prevent the physiological suppression of the inflammasome-mediated activation and, consequently, activate the inflammatory pathways (IL-1β and IL-18) and contribute to ALS pathogenesis (figure 2).[46,49]

	Function
C9orf72[6,21–23,24–28]	Mutations enhance microglial proinflammatory priming, with impaired regulation of the autophagy and lysosomal pathways
CX3CR1[29,30]	Mutations impair a major pathway of microglia-mediated neuroprotection
OPTN[31,32]	Mutations activate the neuroinflammatory NF-κB pathway and impair autophagy
SOD1[19,33,34,35]	Mutations enhance microglial proinflammatory priming
SQSTM1[36,37]	Mutations impair autophagy and increase oxidative stress by altering the phosphorylation of OPTN and SQSTM1/p62
TBK1[38–40]	Mutations impair autophagy by altering the phosphorylation of OPTN and SQSTM1/p62
TARDBP[7,20,41,42]	Mutations enhance microglial proinflammatory priming
TNIP1[43,44]	Mutations activate the NF-κB inflammatory pathway
VCP[45,46]	Mutations impair overall protein degradation by the ubiquitination-proteosome pathway and autophagy

NF-κB=nuclear factor-kappa B.

Table: **Mutant genes that mediate innate immune–inflammatory responses and have been associated with amyotrophic lateral sclerosis**

Inflammation in genetic animal models of ALS

Transgenic rodent models have contributed to our understanding of the pathogenesis of ALS.[34,27] Although these animal models do not completely recapitulate the human disease, they provide valuable insights into neurodegeneration in general and, more specifically, the diversity of cells, cytokines, and chemokines that contribute to the initiation and propagation of the inflammatory responses. These transgenic animal models suggest that ALS is mediated by a non-cell-autonomous process: motoneurons are involved in the initial cell-autonomous injury, but microglia and other non-neuronal subpopulations, such as peripheral T lymphocytes and monocytes, are involved in the non-cell autonomous inflammatory processes and contribute to the pathogenesis of ALS.[33,34] The transgenic models are presented in the order that they were developed, with mutant *SOD1* first, followed by mutant *TARDBP* and *C90rf72*.

SOD1

SOD1 was identified as a causative gene for ALS in humans in 1993.[19] Subsequent transgenic overexpression of different mutant human SOD1 (mSOD1) proteins induced an ALS-like phenotype in mice. Further genetic manipulation of these mice showed that mSOD1 must be expressed in motoneurons, microglia, and astrocytes to produce the ALS-like phenotype.[10,35,41] The mSOD1 is a gain-of-function mutation; unlike *OPTN*, deletion of mouse *SOD1* does not induce an ALS-like disease in mice. In vitro, microglia expressing mSOD1 isolated from transgenic mice were shown to secrete increased pro-inflammatory factors, such as TNF-α, superoxide, and nitric oxide, promoting further motor neuron injury.[7,10,42,50] The trophic and anti-inflammatory factors insulin-like growth factor-1, IL-4, and IL-10 are also released by microglia, and contribute to motoneuron repair and restrict inflammation.[33] In vivo, the activation of microglia is histologically evident in the spinal cords of patients with ALS and transgenic mice.[8]

Activated microglia can exert protective or toxic responses depending on their interaction with the cellular microenvironment and the presence of pathogenic factors (eg, TNF-α, superoxide, and nitric oxide); innate neuroimmune dysfunction is a pathogenic feature of ALS.[33] Evidence that microglia influence disease progression is derived from two studies in which two types of microglia (wild-type and mutant SOD1) were injected in transgenic animals and their effects then compared. The outcomes show that wild-type microglia slow disease progression

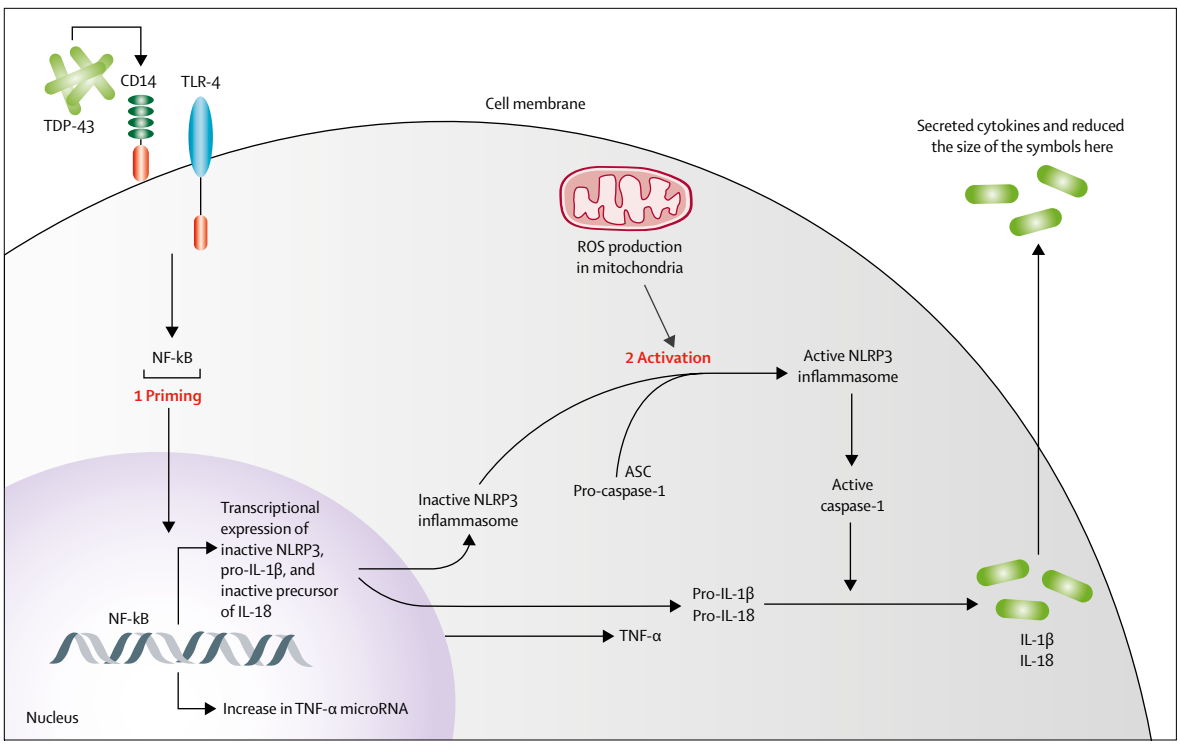

Figure 1: **Model of microglial proinflammatory pathways in amyotrophic lateral sclerosis**
Microglia have a central role in neuroinflammation by releasing proinflammatory cytokines interleukin (IL)-1β and IL-18 in a sequential two-step process. The first step is the priming of the NF-κappa-B (NK-kB) pathway through binding of misfolded aggregated protein TDP-43 to the CD14 on the outer surface of the microglial membrane. In turn the TDP-43–CD14 complex interacts with toll-like receptor (TLR)-4 to initiate the intracellular priming pathway. This step initiates the transcription of proinflammatory genes including pro-IL-1β, pro-IL 18, TNF-α, and inactive precursor form of NLRP3. The second step of the process is the activation of the NLRP3 inflammasome triggered by a diverse number of sensor molecules including mitochondrial reactive oxygen species (ROS). The assembled NLPR3 inflammasome with apoptosis-associated speck (ASC) converts pro-caspase-1 to active caspase-1, cleaving pro-IL-1β and pro-IL-18 to form IL-1β and IL-18, which are then secreted. ASC= apoptosis-associated speck. IL=interleukin. NK-kB=NF-κappa-B. NLRP3=nucleotide-binding domain-like receptor 3. ROS=reactive oxygen species. TLR=toll-like receptor. TNF=tumour necrosis factor.

and prolong survival compared with mSOD1 microglia in *mSOD1-PU.1*$^{-/-}$ transgenic mice; and that deletion of *mSOD1* from wild-type microglia slows disease progression and prolongs survival.[10,41]

TARDBP

Mutations in *TARDBP* have been linked to familial cases of both ALS and frontotemporal dementia, and the presence of ubiquitinated cytoplasmic inclusions of TDP-43 in ALS and frontotemporal dementia prompted the designation of these disorders as TDP-43 proteinopathies.[20] Mutant TDP-43 is present in both motoneurons and glia. Overexpression of mutant TDP-43 promotes a proinflammatory microglia-mediated neurotoxic response and exacerbates TDP-43 proteinopathies in mutant TDP43 transgenic mice.[42]

Pattern recognition and cytokine receptors control the transcription of proinflammatory cytokines interleukin (pro-IL)-1β, whereas the NLRP3 inflammasome regulates the proteolytic processing of pro-IL-1β.[7,51] Signals provided by NF-kB activators are necessary but not sufficient for NLRP3 activation, and a second stimulus is required. In ALS microglia from mSOD1 transgenic mice, the inflammatory process is initiated when TDP-43 binds to CD14 and the complex interacts with toll-like receptor (TLR)-4 and promotes translocation of NF-kB to the nucleus and induction of specific proinflammatory gene expression.[7] This priming step promotes the synthesis of pro-IL-1β and pro-IL-18.[7,23] A second stimulus, such as mitochondrial reactive oxygen species, activates the NLRP3 inflammasome and promotes caspase 1-mediated formation and secretion of mature IL-1β and IL-18, two highly proinflammatory proteins (figure 1).[7]

In a novel TDP-43 mouse model, when human TDP-43 pathology was reversibly expressed in neurons, minimal microglial changes were noted despite increasing motoneuron injury and loss.[53] When human TDP-43 expression was suppressed, the proliferation of microglia increased and cleared the aggregated TDP-43, resulting in functional recovery.[53] The negligible microglial response during neuronal expression of TDP-43 might be associated with the absence of TDP-43 expression in microglia in this mouse model. In other transgenic ALS models and in patients with ALS, TDP-43 is expressed in motoneurons and microglia, and the expression of TDP-43 in microglia can prime the inflammatory

response. Neuroprotection was also noted in the double transgenic *mSOD1/PU.1*$^{-/-}$ mouse; integration (ie, transfusion of bone marrow cells that migrate to the spinal cord and integrate as microglia) of wild-type microglia without mSOD expression prolonged survival compared with integration of mSOD1 microglia.[10] Therefore, wild-type microglia are neuroprotective and slow disease progression whereas mSOD1 microglia are neurotoxic and accelerate disease porgression.

C9orf72

Multiple studies have explored the pathogenic mechanisms of *C9orf72*-mediated diseases.[6,23,54,55] A marked increase in microglial inflammatory activity has been documented in *C9orf72* in patients with ALS and correlates with more rapid disease progression.[6,54] Evidence for a loss of function, a toxic gain-of-function due to sequestration of RNA-binding proteins, and increased dipeptide repeat protein synthesis have all been reported in patients with ALS and *C90rf72* mutations..[24,25,55]

With respect to loss of function, *C9orf72* haploinsufficiency reduces autophagy, increases p62 pathology, and leads to ALS and frontotemporal dementia.[24] Mice without *C9orf72* expression (*C9orf72* knockout mice [*C9orf72*$^{-/-}$]) show impaired regulation of autophagy and lysosomal pathways.[26,27] Endosomal–lysosomal dysfunction is present in peripheral myeloid cells and hyperimmune reactivity is widespread in C9orf72$^{-/-}$ mice.[23,27] Microglia had a proinflammatory phenotype with increased expression of inflammatory cytokines IL-6 and IL-1β.[23] However, C9orf72$^{-/-}$ mice without *C9orf72* expression in motoneurons did not develop motoneuron degeneration or motoneuron disease.[23] Thus, expressing *C9orf72* in innate immune cells, including macrophages and microglia, does not cause motoneuron disease in a transgenic mouse unless *C9orf72* is also expressed in motoneurons. These results resemble the inability of microglia expressing mSOD1 to cause motoneuron injury in PU.1$^{-/-}$ mice.[10] Both the PU.1$^{-/-}$ and C9orf72$^{-/-}$ mouse models suggest that mSOD1 or C9orf72 must be expressed within both motoneurons and microglia to produce the clinical signs of ALS.

The *C9orf72* repeat expansion has differential effects on different cells. A loss of autophagy function in microglia leads to a proinflammatory state and gain-of-function in neurons, and promotes RNA and aggregated protein toxicity (figure 2).[25,28] The fact that systemic inflammation in C9orf72$^{-/-}$ does not lead to motoneuron disease in mice suggests that cell autonomous *C9orf72*-mediated neuronal injury and non-cell autonomous glial and immune-mediated neurotoxicity are needed to promote disease progression and the clinical features of ALS.

Impaired autophagy and enhanced inflammation can result not only from mutations in *C9orf72*, but also in *OPTN*, *TBK1*, *SQSTM1*, *TNIP1*, and *VCP*. Inhibiting autophagy in mSOD1 motoneurons in transgenic mice accelerated neuromuscular junction dysfunction, but

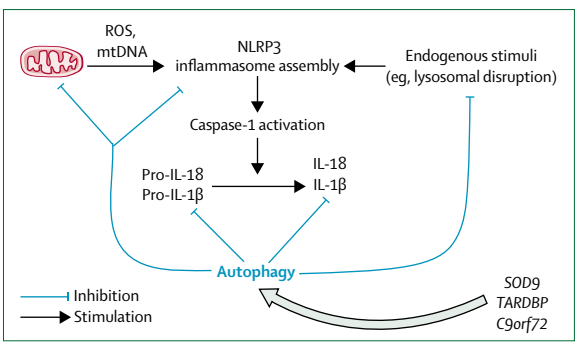

Figure 2: **Interaction of autophagy and inflammasome pathways in amyotrophic lateral sclerosis**
Autophagy restricts the generation of proinflammatory cytokines by inhibiting the NLRP3 inflammasome assembly, mitochondrial production of reactive oxygen species (ROS), DNA, and endogenous stimuli that activate the inflammasome assembly. Autophagy also blocks the production of proinflammatory cytokines interleukin (pro-IL)-1β and pro-IL-18. Disruption of autophagy by mutations in multiple ALS-linked immune genes (grey arrow) prevents inhibition of the inflammasome and thereby enhances the proinflammatory state. IL=interleukin. NLRP3=nucleotide-binding domain-like receptor 3. ROS=reactive oxygen species.

prolonged lifespan.[56] The increased longevity was associated with early reduction in glial inflammation and interneuron pathology. However, blocking autophagy in microglia would have activated the microglial NLRP3 inflammasome and glial inflammation. Thus, blocking autophagy in neurons might have different effects than blocking autophagy in glia. Nevertheless, the fact that multiple genetic mutations alter autophagy and inflammatory signalling provides evidence for the importance of these pathways in the pathogenesis of ALS, but the responses might differ among different cell types. A greater understanding of the interaction of autophagy and activation of the inflammasome pathway in glia versus neurons might offer novel approaches to ALS therapy.

Microglia and astrocytes

Dysregulation of inflammatory pathways is present not only in the 10% of patients with ALS and a positive family history, but also in the 90% of patients with sporadic ALS without a positive family history.[13] Patients with sporadic ALS also have increased inflammation with CNS reactive microglia and astroglia, and activated peripheral monocytes and lymphocytes that infiltrate the CNS. What initiates this immune dysregulation in patients with sporadic ALS is unknown. The inflammatory cytokine IL-6 is secreted from activated macrophages and microglia in transgenic mSOD1 mice and patients with ALS.[2,57,58] Efforts to suppress the microglia-mediated inflammatory response by blocking IL-6 signalling with tocilizumab, a humanised IL-6 receptor antibody, were of no benefit in the mSOD1 mice and of unclear benefit in a pilot trial of ten patients with ALS.[57,58] At baseline, half of the patients with ALS had strong inflammatory activation (ie, inflammatory cytokines measured by ELISA, group 1) and the other half (group 2) had weak activation. Tocilizumab infusions

resulted in downregulation of inflammatory proteins (in particular IL-1β) in group 1 but in an upregulation of inflammatory genes in the group 2.[57] Clinical implications are unclear in these cases because of the mixed results.

Astrocytes also have beneficial and detrimental roles in the pathogenesis of ALS.[3] During the early stages of disease, astrocytes provide a neuroprotective function. As disease progresses, activated astrocytes, activated through either microglia processes or independently by the release of compounds from motoneurons, join activated microglia and release proinflammatory cytokines promoting a neurotoxic environment that contributes to the demise of motoneurons.[3] Thus, inflammatory cytokines released by astrocytes and microglia might facilitate glutamate excitotoxicity thereby linking neuroinflammation and excitotoxic cell death. When a critical threshold is reached, reactive astrocytes and microglia might trigger an irreversible pathological process that subsequently leads to the non-cell autonomous death of motoneurons in patients with ALS.[59]

PET imaging

Until 2010, it was not possible to determine the activation state of microglia without a biopsy or at autopsy. However, development of ligands that bind to activated microglia and astroglia has made PET imaging a valuable technique to monitor inflammation in patients with ALS in real time. Neuroimaging of microglial activation in ALS was first reported by use of the PET ligand [^{11}C]-(R)-PK11195 that binds to translocator protein, previously called the peripheral benzodiazepine receptor (PBR), which is part of a protein complex associated with the outer mitochondrial membrane in microglia and astrocytes.[60] The radioligand [^{11}C]-PBR28 has more translocator protein binding specificity than [^{11}C]-(R)-PK11195.[60] Voxel-based analysis of data acquired [^{11}C]-PBR28 from 53 patients with ALS showed the greatest binding in anatomical regions with the highest disease burden, such as the precentral and paracentral gyri.[61] This study also showed that the greater the binding, the greater the clinical dysfunction in the corticospinal tract (ie, increased spasticity and hyperreflexia). However, uptake and binding of [^{11}C]-PBR28 was not increased despite clinical progression of disease, according to data after 6-month follow-up. Thus, PET imaging with [^{11}C]-PBR28 confirms the presence of glial activation and neuroinflammation in CNS regions that are compromised in patients with ALS and could represent a technique to evaluate the extent of disease burden.

Peripheral inflammation

A study of peripheral blood monocytes from patients with ALS has revealed a proinflammatory phenotype.[2] Whether the peripheral immune myeloid cells enter the CNS and contribute to the development or progression of ALS is still controversial. Embryonic yolk sac macrophages are the main precursors of microglia, whereas most other tissue macrophages are derived from foetal monocytes originated in the bone marrow. The adult tissue-resident microglia and macrophage populations are established from embryonic precursors that arise from these two distinct developmental programmes.[62] However, peripheral myeloid cell engraftment of the CNS can occur following insults that compromise the blood–brain barrier and alter the parenchymal milieu. This engraftment is most evident in the context of neuroinflammatory diseases, such as multiple sclerosis,[63] but blood–brain barrier alterations have also been described in mSOD1 ALS mice and patients with ALS.[64] A compromised blood–brain barrier would allow the entry of activated peripherally derived monocytes or macrophages into the CNS parenchyma of patients with ALS and might contribute to the neuroinflammatory process.

A major limitation in confirming the substantial role of infiltrating monocytes in the neuroinflammatory process has been the absence of specific markers that differentiate CNS microglia from peripheral monocytes. To determine whether peripheral monocytes actually enter the CNS in patients with ALS, specific markers of activated peripheral monocytes that are not present on activated CNS microglia are needed. Monocytes were reported to enter the CNS on the basis of their increased expression of CD169, which was not increased on activated microglia in the same patients.[65]

Microglia have also been reported to have distinct markers that are not present in peripheral macrophages.[66,67] Microglia do not transition through monocytic stages like other tissue-resident macrophages.[68] Transmembrane protein 119 is highly expressed on the surface of microglia in mice and humans, and is not expressed in any peripheral myeloid cells.[66] Monocyte-derived macrophages have a distinct transcriptional profile and can enter the CNS even in the absence of blood–brain barrier breakdown. They maintain their transcriptional profile, which differs from the microglial transcriptional profile.[67] Defining the specific differences in genetic expression of resident CNS microglia from monocyte-derived peripheral macrophages that have migrated to the CNS should help elucidate the potential contribution of these two population of cells (ie, CNS microglia and peripheral macrophages) to inflammation and the pathogenesis of ALS.

Dysfunction in ALS has been proposed to start in the cortex and progress in an anterograde fashion as a so-called dying forward process from corticospinal projections to lower motoneurons.[69] However, most studies report that motoneuron degeneration starts as a distal axonopathy and progresses retrogradely to the anterior horn of spinal cord as a so-called dying back process.[70] Alterations of the neuromuscular junction and skeletal muscle denervation are major determinants of clinical weakness and disease severity in patients with ALS.[70]

The distal motor axons and the neuromuscular junction are outside the blood–brain barrier and have continuous access to circulating myeloid cells and T lymphocytes. In mSOD1 mice, CD68-positive macrophages accumulate

within peripheral nerves but whether they are protective or contribute to disease progression has not been elucidated.[71] A transgenic mouse model of ALS suggests that the scarcity of MHC I and CD8 T lymphocytes impaired the neuromuscular junction (because MHCI and CD8 T lymphocytes accelerate the removal of nerve axon debris, which then encourages axonal regrowth and nerve terminal sprouting and enhances neuromuscular structure and function), yet prolonged survival.[72] The presence of MHC I and CD8 T lymphocytes promoted axonal regrowth and preserved muscle innervation.[72] These results suggest that the peripheral inflammatory response can be neuroprotective while the CNS is under a proinflammatory siege.

The neuromuscular junction was also the focus of a therapeutic trial in patients with ALS using ozanezumab, a monoclonal antibody against the neurite outgrowth inhibitor A (Nogo-A), which is upregulated in the muscle of these patients and prevents repair of the neuromuscular junction.[73] Unfortunately, this phase 2, randomised, placebo-controlled clinical trial showed no evidence of efficacy, suggesting that the increase in Nogo-A might not have a specific role in the pathophysiology of ALS.

Emerging cell-based therapies

Clinical investigations of cell-based therapies have been initiated in patients with ALS with the rationale that cells—whether mesenchymal or progenitor stem cells—might enhance neuronal repair or suppress neurocytotoxic activity. Although these studies might have promising results, they are in the early stages and have yet to provide convincing evidence of efficacy.

Mesenchymal stem cells derived from bone marrow or fat have been infused into the CSF of patients with ALS in several phase 1 clinical studies.[74–78] Intrathecal administration is apparently more efficacious than other routes of administration, and single intrathecal infusions of bone marrow-derived mesenchymal stem cells have been safe and well tolerated, with no serious adverse events, in 14 patients with ALS in a phase 2a clinical trial (NCT01777646).[74] The potential therapeutic benefit has been attributed to increased concentrations of neurotrophic growth factors (eg, glial-derived neurotrophic factor, brain-derived neurotrophic factor, VEGF) available to repair motoneurons and to the suppression of neuroinflammation from increased secretions of anti-inflammatory cytokines. Two intrathecal injections of bone marrow-derived mesenchymal stem cells 26 days apart were also safe and well tolerated in patients with ALS.[75,76] Intrathecal administration of fat-derived mesenchymal stem cells produced lumbosacral-radicular pain and dose-dependent changes in CSF protein.[77] All these effects were reported as temporary, tolerable, and otherwise safe. Although the potential benefits have been attributed to enhanced neurotrophic growth factor secretion, neurotrophic growth factors have not proven beneficial when administered individually to patients with ALS.[79] Mesenchymal stem cells-mediated

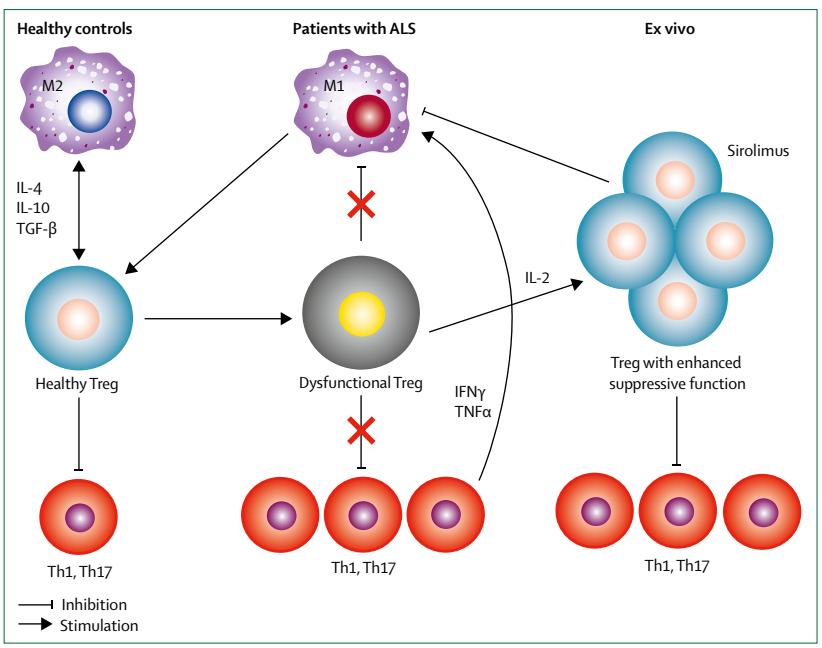

Figure 3: **Hypothetical model of immune cell interactions in amyotrophic lateral sclerosis**
In healthy individuals, regulatory T cells (Tregs) and macrophages (M2) release the anti-inflammatory cytokines interleukin (IL)-4, IL-10, and transforming growth factor (TGF)-β. Healthy Tregs suppress proinflammatory helper T (Th)1 and Th17 lymphocytes, thus reinforcing the anti-inflammatory status. In patients with amyotrophic lateral sclerosis (ALS), macrophages are proinflammatory (M1) and promote conversion of healthy Tregs to dysfunctional Tregs, which can no longer suppress Th1 and Th17 lymphocytes, thereby enhancing the release of interferon (IFN)-γ and tumour necrosis factor (TNF)-α, further activating M1. The relative proinflammatory balance is associated with fast disease progression. Ex vivo studies have shown that expansion of the dysfunctional Tregs with IL-2 and sirolimus, besides promoting an increase in the number of Tregs, also restores their ability to suppress Th1 and Th17 lymphocytes, and also suppresses M1.[82] Autologous administration of the expanded Tregs has been associated with slowed clinical progression in patients with ALS.[82] ALS=amyotrophic lateral sclerosis. IFN= interferon. IL=interleukin. M=macrophages. TGF=transforming growth factor. Th=T-helper cells. TNF= tumour necrosis factor. Tregs= regulatory T cells.

stimulation of regulatory T cells (Tregs) populations might be the major mechanism of action of the potential benefits.[78] Tregs suppress both pro-inflammatory T effector lymphocytes and activated macrophages and microglia (figure 3), but in ALS Tregs are dysfunctional and their anti-inflammatory suppressive functions are substantially lessened.[80] Although mesenchymal stem cells are safe and well tolerated, several questions remain in addition to clinical efficacy, such as how long do the cells persist, how long do their functional effects last, how frequently will infusions be necessary, and what is the evidence for target engagement.

Neural progenitor cells are multipotent cells committed to the neural cell lineage that can self-renew and be readily expanded in vitro. In an open-label phase 2 clinical trial (NCT01730716) of 15 patients with ALS previously treated with immunosuppressants, neural progenitor cells were directly injected into the cervical and lumber spinal cord of these patients.[81] Results of the postoperative ALS Functional Rating Score Revised and forced vital capacity slopes of transplanted patients did not differ from those of three separate historical control groups. Therefore, the stem cell transplantation did not seem to benefit the

Search strategy and selection criteria

We searched for articles published in English on PubMed between Dec 1, 2013, and Oct 10, 2018, with the search terms "ALS AND genetics", "motoneuron disease AND genetics", "ALS AND mutation", "ALS AND inflammation", "ALS AND neurodegeneration", "ALS AND microglia", "ALS AND astrocytes", "ALS AND monocyte", "ALS AND macrophage", and "ALS AND lymphocytes." We selected articles that reported the identification of the ALS genes *SOD1*, *TARDBP*, *C9orf72*, *OPTN*, *TBK1*, *SQSTM1*, *TNIP1*, *VCP*, and *CX3CR1*. We also searched for articles describing the function and implications of mutations in these selected genes in neurological and non-neurological diseases, and associations with known ALS genes. We selected the most relevant articles on the basis of our subjective appraisal of their quality and mechanistic insight that could be relevant to this Review.

patients. Adverse events were mostly related to transient pain associated with surgery and to side-effects of the immunosuppressant medications.

Despite increasing evidence for the role of altered immunity in animal models of ALS and patients with ALS, administration of immunosuppressive or immunomodulatory drugs have not altered the progression of the disease.[82] The absence of benefit has been attributed to the heterogeneity of disease, resulting in difficulty to administer immunomodulatory therapy within an appropriate time frame. However, an alternative explanation is that drug immunotherapy equally targets proinflammatory T-helper (Th)1 and Th17 cells and anti-inflammatory Tregs, and thus does not change their relative balance and functional contributions. Effective therapy should increase Tregs without increasing Th1 and Th17 cells, or suppress Th1 and Th17 without targeting Tregs, thereby enhancing anti-inflammatory responses. An appropriate strategy might be to increase Tregs by intravenous infusions.

Tregs have been shown to affect disease progression and survival in ALS mouse models and in patients with ALS.[8,9,83] In patients with ALS, inflammation is associated with decreased numbers of Tregs and decreased FOXP3 mRNA expression, the main transcription factor in the development and function of Tregs, which results in accelerated disease progression.[9,83] Lower concentrations of Tregs in patients with ALS are associated with increased mortality, whereas higher concentrations are associated with longer survival.[9] Tregs in patients with ALS are dysfunctional, with impaired suppression of responder T lymphocyte (Tresp) proliferation.[80] Expansion of the dysfunctional Tregs ex vivo restores their ability to suppress Tresp proliferation (figure 3).[84] Autologous intravenous infusions of these expanded Tregs into three patients with ALS formed the basis of a first-in-human pilot study of safety and tolerability of this intervention.[84] These infusions were safe and well tolerated and, during infusions, clinical progression was slowed and inspiratory pressure stabilised (time of infusion varied among studies). The suppressive function of the Tregs assayed ex vivo (from cells extracted at different disease stages) was correlated with the clinical state: Tregs suppressive function increased during slow clinical progression, and suppressive function decreased during increased clinical progression.[84] A question for future studies is whether placebo-controlled administration of Tregs at regular intervals can prolong the slowing of disease progression, and thus benefit a larger cohort of patients with ALS.

Conclusions and future directions

Mutations in multiple genes that are expressed in both neurons and microglia give rise to ALS and frontotemporal dementia by impairing autophagy, enhancing microglial and astroglial inflammatory pathways, and promoting motoneuron cell death.[13,49,55] These ALS-linked mutant immune genes provide evidence that immune dysregulation contributes to the pathogenesis of ALS. Efforts to reverse the dysfunction in autophagy represent an important therapeutic goal. Even patients with sporadic ALS without known genetic abnormalities present with immune dysregulation characterised by increased proinflammatory macrophages and dysfunctional Tregs.[2,80] In patients with ALS, microglia and astrocytes are activated and neuroprotective early in the course of disease and proinflammatory in later stages. What initiates this immune dysregulation in sporadic ALS is unclear, but the consequences are enhanced neuroinflammation, impaired motoneuron function, and fast disease progression. In transgenic animal models of ALS, during the early stages of disease, activated microglia and astroglia are anti-inflammatory and protect motoneurons, but in later stages, as disease progresses, accelerated motoneuron injury is mediated by misfolded proteins that promote the release of damage-associated molecular pattern signalling, and consequently activate a proinflammatory glial cascade. Identification of motoneuron–glial signalling is an important priority for developing new therapeutic strategies. Cell-based strategies that enhance the anti-inflammatory reactivity and reverse immune dysregulation offer the potential of slowing disease progression and improving quality of life of patients with ALS.

An important future direction is the development of effective ways to monitor inflammatory-mediated disease pathophysiology and progression. Advances in neuroimaging, especially PET, to determine the activated state of astrocytes and microglia in brains and spinal cords of patients with ALS, and their relative contribution to disease pathophysiology and progression are promising. However, specific ligands whose binding increases with disease progression and severity, and ligands that can differentiate anti-inflammatory from proinflammatory microglia need to be developed. Technological limitations of imaging the spinal cord, the main site for inflammatory pathology, also need to be overcome. Biomarkers that can effectively

monitor the inflammatory phenotypes of peripheral macrophages and T lymphocytes are also needed. The suppressive function of Tregs was associated with the clinical state of patients with ALS in a small study of autologous infusion of expanded Tregs;[84] defining the specific molecules mediating such suppression might provide a meaningful therapeutic target to slow disease progression.

Contributors

Both authors contributed equally to the literature search, collection of reported data, interpretation of data, organisation and writing of the review, and design of the figures.

Declaration of interests

DRB is a scientific consultant to Implicit. SHA is a scientific consultant to Mitsubishi Tanabe Pharma, Neuraltus, Implicit, and Brainstorm; received speaker honoraria from and was the speaker's bureau of Avanir; and received research support from the Amyotrophic Lateral Sclerosis Association, Amyotrophic Lateral Sclerosis Finding a Cure, and Muscular Dystrophy Association.

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