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REVIEW ARTICLE

Immunity in amyotrophic lateral sclerosis: blurred lines between excessive inflammation and inefficient immune responses

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Despite wide genetic, environmental and clinical heterogeneity in amyotrophic lateral sclerosis, a rapidly fatal neurodegenerative disease targeting motoneurons, neuroinflammation is a common finding. It is marked by local glial activation, T cell infiltration and systemic immune system activation. The immune system has a prominent role in the pathogenesis of various chronic diseases, hence some of them, including some types of cancer, are successfully targeted by immunotherapeutic approaches. However, various anti-inflammatory or immunosuppressive therapies in amyotrophic lateral sclerosis have failed. This prompted increased scrutiny over the immune-mediated processes underlying amyotrophic lateral sclerosis. Perhaps the biggest conundrum is that amyotrophic lateral sclerosis pathogenesis exhibits features of three otherwise distinct immune dysfunctions—excessive inflammation, autoimmunity and inefficient immune responses. Epidemiological and genome-wide association studies show only minimal overlap between amyotrophic lateral sclerosis and autoimmune diseases, so excessive inflammation is usually thought to be secondary to protein aggregation, mitochondrial damage or other stresses. In contrast, several recently characterized amyotrophic lateral sclerosis-linked mutations, including those in *TBK1*, *OPTN*, *CYLD* and *C9orf72*, could lead to inefficient immune responses and/or damage pile-up, suggesting that an innate immunodeficiency may also be a trigger and/or modifier of this disease. In such cases, non-selective immunosuppression would further restrict neuroprotective immune responses. Here we discuss multiple layers of immune-mediated neuroprotection and neurotoxicity in amyotrophic lateral sclerosis. Particular focus is placed on individual patient mutations that directly or indirectly affect the immune system, and the mechanisms by which these mutations influence disease progression. The topic of immunity in amyotrophic lateral sclerosis is timely and relevant, because it is one of the few common and potentially malleable denominators in this heterogenous disease. Importantly, amyotrophic lateral sclerosis progression has recently been intricately linked to patient T cell and monocyte profiles, as well as polymorphisms in cytokine and chemokine receptors. For this reason, precise patient stratification based on immunophenotyping will be crucial for efficient therapies.

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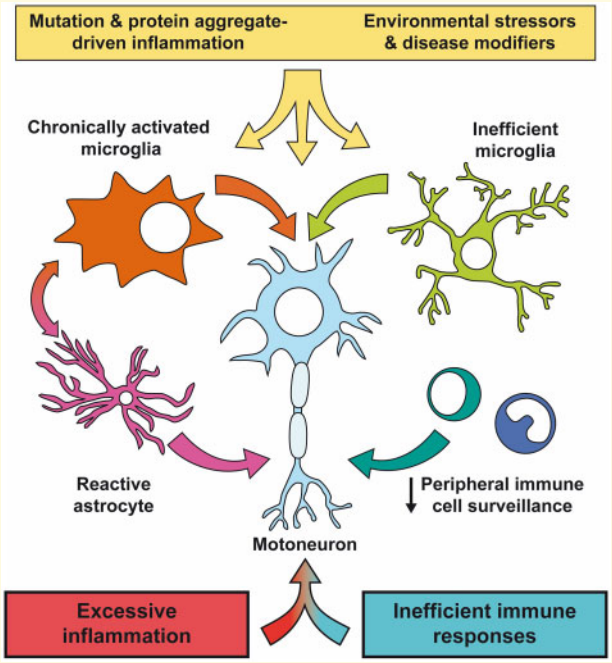
Abbreviations: ABIN1 = A20-binding inhibitor of NF-κB; ALS = amyotrophic lateral sclerosis; ALSFRS-R = Amyotrophic Lateral Sclerosis Functional Rating Scale – Revised; BBB = blood–brain barrier; BDNF = brain-derived neurotrophic factor; CD = cluster of differentiation; CHIT1 = chitinase 1; CHI3L1 = chitinase 3-like 1; CHI3L2 = chitinase 3-like 2;CX3CL1 = fractalkine; CX3CR = CX3C chemokine receptor 1 or fractalkine receptor; CYLD = cylindromatosis; C9orf72 = chromosome 9 open reading frame 72 gene; DAM = disease-associated microglia; DAMP = damage-associated molecular patterns; DC = dendritic cells; EAAT2 = excitatory amino acid transporter 2; FUS = fused in sarcoma; HMGB1 = high-mobility group box 1; hSOD1 = human supeoxide dismutase 1; HTLV-1 = human T cell leukaemia virus-1; IFN = interferon; IGF1 = insulin-like growth factor 1; IgG = immunoglobulin G; IL = interleukin; IKK = IκB kinase; IRF = IFN regulatory factor; LPS = lipopolysaccharide; MAPK = mitogen-activated protein kinases; MyD88 = myeloid differentiation factor 88; NEFM = neurofilament medium polypeptide; NK = natural killer cells; NF-κB = nuclear factor of κB; OPTN = optineurin; PAMP = pathogen-associated molecular patterns; RAGE = receptors for advanced glycation end products; RNS = reactive nitrogen species; ROS = reactive oxygen species; SOD1 = superoxide dismutase 1; SRSF3 = serine/arginine-rich splicing factor 3; TBK1 = TANK-binding kinase 1; TDP-43 = transactive response DNA-binding protein of 43 kDa; TGF = transforming growth factor; TLR = Toll-like receptors; Tmem119 = transmembrane protein 119; TNF = tumour necrosis factor; TRAIL = TNF-related apoptosis-inducing ligand; Treg = regulatory T cells; TREM2 = triggering receptor expressed on myeloid cells 2; TRIF = TIR-domain-containing adapter-inducing IFN-β; YM1 = chitinase-like protein 3; WT = wild type

Graphical Abstract



Introduction

Chronic inflammation is considered to be a key contributing factor to diseases as diverse as cancer, stroke, heart disease, obesity and depression. It is also an early and common hallmark of neurodegenerative diseases (Ransohoff, 2016). As the only immune cell subtype residing in the brain parenchyma, microglia are tasked with orchestrating inflammation against neurotropic infectious agents as well as clearing the debris in stroke and neurodegenerative diseases. Nevertheless, when Alois Alzheimer described reactive cells surrounding amyloid β plaques in the first case of his namesake disease in the early 1900s, the exact function of these cells was unclear (Alzheimer, 1911; Kettenmann *et al.*, 2011). Microglia were subsequently identified by Pío del Río Hortega in 1919, who also described their phagocytic capacity and reactivity in response to external stimuli (R$_i$o-Hortega, 1919*a*, *b*; Sierra *et al.*, 2016). However, it took another 70 years until it was recognized that activated microglia are key elements not only in Alzheimer's disease but also in amyotrophic lateral sclerosis (ALS) and other

neurodegenerative diseases (McGeer *et al.*, 1987, 1988; McGeer and McGeer, 2011). Around the same time, the infiltration of T cells was first reported in ALS patients (Troost *et al.*, 1989; Engelhardt *et al.*, 1993). These findings strengthened the notion that glial activation and peripheral immune cell infiltration in the brain is neurotoxic and contributes to ALS, as it was previously reported for multiple sclerosis and spinal cord injury (DiSabato *et al.*, 2016; Ransohoff, 2016). Hence, it came as both surprise and disappointment that a large number of ALS clinical trials found no protection provided by various anti-inflammatory agents, similar to trials in other neurodegenerative diseases and traumatic brain injury (Adapt-Fs, 2015; Russo and McGavern, 2016; Petrov *et al.*, 2017). Multiple reasons for these failures likely exist. Firstly, despite the well-known neurotoxic effects of inflammation, non-specific downregulation of the immune system limits neuroprotective and neuroreparative functions of CD4 T cells and microglia (Schwartz and Shechter, 2010; Russo and McGavern, 2016). Secondly, ALS is a phenotypically and genetically heterogenous disease, so effective treatment necessitates stratified case monitoring (Goyal *et al.*, 2020). Finally, immunological biomarkers are necessary for precise tracking of immune responses from preclinical to the end disease stages, thus guiding precision medicine.

This review discusses the complex role of inflammation and immunity during the course of ALS and details the functions of individual immune cell subsets during disease progression. We can nowadays firmly renounce the long-held dogma that the CNS remains safe by keeping the immune system off-limits (Schwartz and Deczkowska, 2016). Dural lymphatic vessels are now known to drain to deep cervical lymph nodes (Aspelund *et al.*, 2015; Louveau *et al.*, 2015). Furthermore, T cells, monocytes and other peripheral immune cells can directly access and/or indirectly influence the CNS via the choroid plexus (Baruch *et al.*, 2014). Therefore, the longstanding perception of the brain as a strictly immunoprivileged organ is challenged by striking examples of active immune surveillance of the brain in both health and disease, suggesting that the main function of the immune system in the CNS is maintaining homeostasis (Medawar, 1948; Ziv *et al.*, 2006; Aguzzi *et al.*, 2013; Deczkowska *et al.*, 2018*a*). However, in response to excessive or prolonged CNS damage, the protective functions of the immune system are thought to break down and lead to hyperinflammation, neuronal damage and unrelenting ALS progression. Moreover, in the recent years, a growing body of evidence has indicated that deregulation of immune response is not solely an aftermath of neuronal death, and that it may in many cases occur early in the course of ALS. This was corroborated by the longitudinal bioluminescence live imaging studies in mouse ALS models that revealed marked changes in activation of astrocytes and microglia in the very early presymptomatic stages of disease (Keller *et al.*, 2011; Swarup *et al.*, 2011*a*; Gravel *et al.*, 2016). Furthermore, mutations in several genes directly linked to the immune response have recently been reported in ALS patients (Maruyama *et al.*, 2010; CirulLi *et al.*, 2015; Freischmidt *et al.*, 2015; Dobson-Stone *et al.*, 2020). Some of them could lead to inefficient immune responses rather than overt hyperinflammation, adding to the complexity of immune system dysfunctions in ALS. Therefore, we propose that the loss of immune homeostasis and/or early deregulation of immune responses may represent one of a key initial steps in disease pathogenesis leading to either excessive inflammation or an inefficient immune response (immunodeficiency), either of which could contribute to ALS progression. Due to the dual role of the immune system, patient stratification based on their immune profiles, and precise timing and/or specific targeting of excessive inflammation while maintaining the protective and regenerative immune functions, will likely be crucial for the efficient treatment of neurodegenerative diseases.

ALS is a systemic and immune-mediated disease

Commencing with Jean-Martin Charcot, considerable efforts have been made to correlate clinical symptoms to neuroanatomical findings, linking each neurodegenerative disease to discrete sets of damaged neurons (Kumar *et al.*, 2011). However, most neurodegenerative diseases have systemic effects, clinically overlap and share common immunopathogenic mechanisms. In ALS, up to 50% of ALS patients have cognitive problems, some of which exhibit overt symptoms of frontotemporal dementia (Hardiman *et al.*, 2017). Moreover, the same hexanucleotide repeat expansion in chromosome 9 open reading frame 72 gene (*C9orf72*), which is the most frequent genetic cause of ALS, may in some family members cause predominant frontotemporal dementia, suggesting that these disorders present the opposite ends of a clinical spectrum (Ling *et al.*, 2013). The systemic aspect of ALS is perhaps best recognized in the seemingly pure motoric ALS cases, in which an accompanying inflammatory response is not restricted to the vicinity of motoneurons, and is detected in muscles, peripheral motor fibres, skin, liver and blood as well (Ono *et al.*, 2001; Henkel *et al.*, 2004; Chiu *et al.*, 2009; Nardo *et al.*, 2016; Gasco *et al.*, 2017; Paré and Gros-Louis, 2017). An underlying immune component may contribute to delaying neurodegeneration in the individuals carrying highly penetrant familial mutations. Indeed, although late onset ALS is generally linked to an age-related deterioration of intrinsic motoneuron pathways (Khalil *et al.*, 2018; Loeffler, 2019), immune senescence and proinflammatory skewing linked to the aging process (sometimes referred to as inflammaging) may also contribute to disease progression (Franceschi *et al.*, 2000; Deleidi *et al.*, 2015; Franceschi

et al., 2018). Notably, once ALS symptoms appear, the progression towards the fatal outcome is extremely rapid, faster than in any other neurodegenerative disease, and in some cases, a rapid progression has been directly linked to immune parameters, which will be further discussed in subsequent chapters (Lopez-Lopez *et al.*, 2014; Wosiski-Kuhn *et al.*, 2019*b*).

ALS genetics and mechanisms

Since the discovery of the first ALS mutation in 1993 in superoxide dismutase 1 (*SOD1*), more than 50 different genes have been linked to ALS (Rosen *et al.*, 1993; Renton *et al.*, 2014; Brown and Al-Chalabi, 2017; Hardiman *et al.*, 2017; Mejzini *et al.*, 2019). Their exact roles in disease pathogenesis are still incompletely understood. The main proposed mechanisms include impaired proteostasis (enhanced protein aggregation and/or decreased degradation), dysregulated RNA metabolism, blockade of nucleocytoplasmic transport, mitochondrial dysfunction and impaired axonal transport. Thus, although inflammation is a common denominator in various ALS aetiologies, most ALS mutations do not directly target the immune system. However, proteins and/or peptides encoded by mutations in *SOD1*, *TARDBP*, *FUS* and *C9orf72* gain propensity to form aggregates, which in turn trigger inflammatory responses in microglia (Fig. 1). Aggregates also form in sporadic ALS patients with unknown genetic background and contain wild type (WT) transactive response DNA-binding protein of 43 kDa (TDP-43, encoded by *TARDBP*). Notably, the crosstalk between protein aggregation and inflammation is bidirectional, and in inflammatory settings, nuclear TDP-43 gets phosphorylated, mislocalized and aggregated in the cytoplasm (Correia *et al.*, 2015; Xue *et al.*, 2018*b*). TDP-43 was also previously reported as an interacting partner and co-activator of p65, a subunit of nuclear factor of κB (NF-κB), the master transcription factor regulating proinflammatory factor production (Swarup *et al.*, 2011*b*). Aggregation of ubiquilin 2 (encoded by *UBQLN2* gene) stimulates TDP-43 co-aggregation and NF-κB activation (Picher-Martel *et al.*, 2015). Another DNA/RNA-binding protein, fused in sarcoma (FUS) also acts as a co-activator of NF-κB, and expression of disease-associated *FUS* mutation (R521G) in astrocytes led to a tumour necrosis factor (TNF)-induced motoneuron death, which could be rescued by NF-κB inhibition (Uranishi *et al.*, 2001; Kia *et al.*, 2018). Moreover, viral infection causes mutant FUS aggregation (Shelkovnikova *et al.*, 2019). In addition to these aggregate-prone proteins, numerous other genes mutated in ALS such as *p62/SQSTM1*, *TBK1*, *OPTN*, *ALS2*, *CHMP2B*, *C9orf72* and *CYLD* were reported to disrupt proteostasis at the level of proteasomal or autophagosomal degradation, and could therefore indirectly facilitate aggregation and trigger inflammation

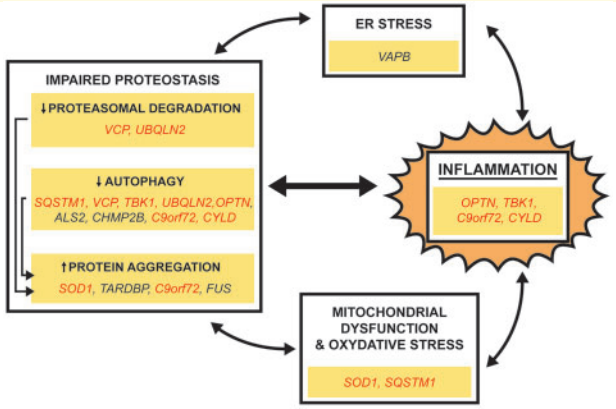


Figure 1 All roads lead to inflammation: genetic evidence. Mutations in a small subset of genes (*OPTN*, *TBK1*, *CYLD* and *C9orf72*) are directly linked with dysfunctional inflammatory responses. Most genes mutated in ALS disrupt proteostasis by increased protein aggregation, decreased proteasomal degradation or impaired autophagy. Aggregated proteins can in turn trigger inflammation. Inflammation can also be triggered by mutations in *VAPB* that causes endoplasmic reticulum stress or mutations in *SOD1* and *SQSTM1* that cause mitochondrial damage and oxidative stress. The link between inflammation and other proposed ALS-causing mechanisms is bidirectional: enhanced inflammation amplifies other pathogenic mechanism. Remark: genes marked in red are proposed act by more than one pathogenic mechanism.

(Fig. 1). Mitochondrial damage, endoplasmic reticulum stress response, and several other mechanisms also promote inflammation, and can at some point initiate a vicious cycle that leads to neurodegeneration. Therefore, inflammation, which is capable of exerting collateral damage, is linked to various aggregate-prone mutations, stress stimuli and/or defects in aggregate disposal.

The possibility that the immune system disbalance can also be an initial ALS trigger or a key disease modifier is only now taking hold. This has initially been dismissed because immune cell activation was reported to arise after the initial neuronal damage (Kano *et al.*, 2012). Moreover, ALS was reported to be only slightly more frequent in patients with a prior diagnosis of autoimmune diseases (Turner *et al.*, 2013), and recent genome-wide association studies found no strong association between ALS and autoimmune diseases (Broce *et al.*, 2018). However, a subset of ALS-linked genes, such as *TBK1*, *OPTN*, *CYLD* and *C9orf72*, encode for multifunctional proteins involved in innate immune reactions. Moreover, polymorphisms in cytokine and chemokine receptors were shown to influence disease progression (Lopez-Lopez *et al.*, 2014; Calvo *et al.*, 2018; Wosiski-Kuhn *et al.*, 2019*b*). Therefore, disbalanced immune responses leading to either excessive inflammation or inefficient immune response (immunodeficiency) may occur early during disease progression or serve as a disease modifier in ALS pathogenesis.

Glial cells as key contributors to ALS pathogenesis

The proposed passive role of glia as the glue that holds neurons together, has been firmly abandoned (Somjen, 1988; Ban *et al.*, 2019). Glial cells actively maintain neuronal homeostasis but also take key roles in the neurodegenerative process (Ransohoff, 2016). Microglia represent the resident innate immune cells of the CNS, separated from peripheral populations during the early embryonic development, without a possibility of being replenished from bone marrow precursors (Ajami *et al.*, 2007; Ginhoux and Prinz, 2015). They survey neuronal microenvironment with their remarkably motile processes and secrete various neurotrophic factors (Fig. 2A) (Davalos *et al.*, 2005; Nimmerjahn *et al.*, 2005; Kettenmann *et al.*, 2013). The neighbouring healthy neurons mitigate microglial activation by negative co-stimulatory molecule CD200 and inhibitory chemokine fractalkine (CX3CL1), whereas astrocytes provide anti-inflammatory cytokine transforming growth factor β (TGF-β) (Fig. 2A) (Hellwig *et al.*, 2013; Butovsky *et al.*, 2014). Astrocytes provide major structural and trophic support to neurons, supplying them with nutrients, growth factors and maintaining the blood–brain barrier (BBB) (Verkhratsky *et al.*, 2013; Liddelow and Barres, 2017). Although they are not considered to be immune cells *per se*, in response to proinflammatory cytokines from activated microglia they become reactive i.e. they proliferate and secrete proinflammatory cytokines (Welser-Alves *et al.*, 2011; Liddelow *et al.*, 2017).

The data supporting a decisive role of non-neuronal cells in ALS are copious. Perhaps the strongest proof that motoneuron death in ALS is primarily non-cell autonomous came from analysing the mice carrying highly penetrant human *SOD1* (*hSOD1*) mutations in neurons (Pramatarova *et al.*, 2001; Lino *et al.*, 2002). Unlike the ubiquitous expression of mutant hSOD1 in a mouse model, which leads to rapidly progressive disease and premature death, selective transgene expression in neurons or motoneurons fails to precipitate symptoms or death, strongly demonstrating that mutation-carrying glia contribute to neuronal pathology. Similarly, the disease could be alleviated in chimeric mutant hSOD1 mice when the diseased neurons were surrounded by healthy glia (Clement, 2003). Selective deletion of mutant *hSOD1* transgene in motoneurons delays the disease onset, but provides no protection from disease progression and death, demonstrating that glial cells carrying the mutant *hSOD1* transgene are sufficient to trigger motoneuron death (Boillee *et al.*, 2006). Individual glial subsets from mutation-carrying mice have distinct neurotoxic potentials by themselves. Specifically, astrocytes carrying $hSOD1^{G93A}$ elicit motoneuron degeneration and microgliosis, whereas reconstitution of microglia-deficient ($PU1^{-/-}$) mice with $mSOD1^{G93A}$ microglia did not result in motor weakness (Beers *et al.*, 2006; Papadeas *et al.*, 2011). These findings suggest that affected astrocytes can trigger motoneuron degeneration by themselves, while microglia require crosstalk with other diseased cells. In contrast, specific deletion of mutant *hSOD1* transgene in the myeloid lineage (targeting both microglia and macrophages) or in astrocytes does not alter disease onset, but slows disease progression and increases life span (Boillee *et al.*, 2006; Yamanaka *et al.*, 2008). Experimental reconstitution with WT myeloid cells was also researched as a potential therapeutic intervention in microglia-deficient mutant hSOD1 mice (Beers *et al.*, 2006). The reconstitution with the WT but not mutant SOD1 bone marrow extends the life span.

It was recently noted that glial activation differs between ALS models. In contrast to extensive activation in SOD1 models, mouse models carrying patient-derived mutations or haploinsufficiency of TANK-binding kinase 1 (TBK1) and optineurin (encoded by *OPTN* gene) lack overt microgliosis and astrocytosis (Ito *et al.*, 2016; Gerbino *et al.*, 2020). Similarly, in a model in which TDP-43 pathology can be reversibly induced in neurons, microglia are only subtly activated (Spiller *et al.*, 2018). However, upon cessation of transgenic TDP-43 expression, microglia get robustly activated and clear the aggregated TDP-43 from the surviving neurons, suggesting that their function is inhibited by damaged neurons. Similar divergence in microglial phenotypes was found in ALS patients, whereby patients carrying *SOD1* mutations had higher microglial activation than *C9orf72* mutation carriers or sporadic ALS patients (Spiller *et al.*, 2018). In contrast, another study found that *C9orf72* mutation carriers had higher microglial activation than sporadic patients (Brettschneider *et al.*, 2012), highlighting the considerable variability between patients.

Several ALS-related phenotypes have been recently reproduced in glial cells derived from patient-induced pluripotent stem cells, adding to the breadth of ALS models. Astrocytes derived from both sporadic and familial ALS patient-induced pluripotent stem cells have been shown to trigger necroptotic cell death of motoneurons in co-cultures (Re *et al.*, 2014). Interestingly, SOD1 and TDP-43 were dispensable in this process, and death was triggered by soluble toxic factors. Another report showed that induced pluripotent stem cell-derived astrocytes from *C9orf72* patients exerted motoneuron toxicity that correlated with elevated astrocytic oxidative stress, reduced antioxidant capacity and senescence (Birger *et al.*, 2019). Interestingly, both necroptosis and oxidative stress have a potential to boost an inflammatory response, which can further increase astrocytic neurotoxicity. In contrast, motoneurons derived from $TDP\text{-}43^{M337V}$ patients showed increased cell-autonomous susceptibility to cell death, and co-culture with induced pluripotent stem cell-derived astrocytes—perhaps counterintuitively—increased their survival (Bilican *et al.*, 2012; Serio *et al.*, 2013). The

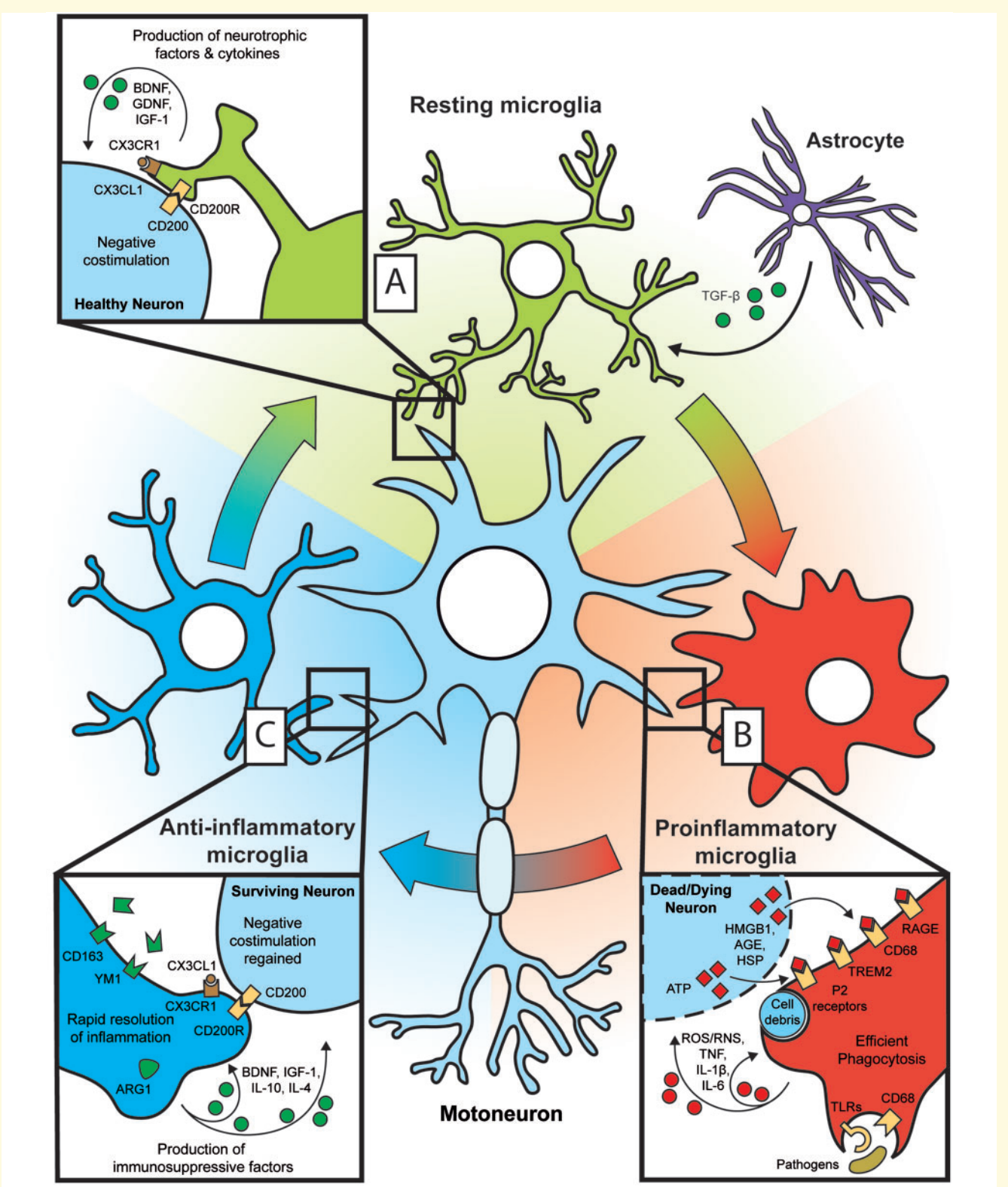


Figure 2 Acute neuroinflammation. **(A)** Microglia maintain homeostasis in the CNS by secreting neurotrophic factors (BDNF, GDNF, IGF-1). Microglial activation is suppressed by healthy neurons that provide negative co-stimulation via CD200 and CX3CL1, and astrocytes that secrete TGF-β. **(B)** Various PAMPs/DAMPs from dead/dying neurons and/or pathogens switch microglia from resting to proinflammatory state by binding to various pathogen and scavenger receptors. Such microglia clear cellular debris, produce proinflammatory factors (TNF, IL-1β, IL-6, ROS/RNS) and upregulate various receptors. **(C)** Upon damage resolution, the remaining neurons restore negative CD200 and CX3CL1 co-stimulation so microglia shift to an anti-inflammatory phenotype. Such microglia secrete anti-inflammatory cytokines IL-10 and IL-4, growth factors, and upregulate arginase 1, YM-1 and CD163, which leads to the resolution of inflammation and restoration of the resting state.

observed discrepancies suggest a marked diversity in individual mutation-specific phenotypes. To conclude, the findings listed in this chapter, demonstrate that not only motoneurons but also glia can be intrinsically affected by the misfolded proteins, and that in ALS motoneurons in many cases die in a non-cell-autonomous manner. The particularities between the extent of glial activation in diverse genetic backgrounds are likely to be crucial for predicting prognosis, monitoring disease progression and determining personalized therapeutic approaches.

Acute versus chronic neuroinflammation

Damage sensing

Microglia are the first responders to damage in the CNS (Fig. 2B) (Kettenmann *et al.*, 2011; Heneka *et al.*, 2014; Roers *et al.*, 2016). Pathogen-associated molecular patterns (PAMPs) such as microbial DNA, RNA, lipopolysaccharide (LPS), and endogenous damage-associated molecular patterns (DAMPs), such as aggregated proteins, high-mobility group box 1 (HMGB1) and heat shock proteins (HSP-60 and -70), bind to similar receptors to activate microglia. For example, LPS and ALS-specific aggregates of mutated SOD1 and TDP-43 bind to Toll-like receptors (TLR)-2 and 4 (Roberts *et al.*, 2013; Zhao *et al.*, 2015). Numerous other damage sensors might be important for microglial activation in neurodegenerative diseases including complement receptors, P2 purinergic receptors (for ATP), receptors for advanced glycation end products (RAGE), inflammasomes and triggering receptor expressed on myeloid cells 2 (TREM2) (Davalos *et al.*, 2005; Lee *et al.*, 2013; Heneka *et al.*, 2014; Juranek *et al.*, 2015; Debye *et al.*, 2018; Deczkowska *et al.*, 2018*b*). Importantly, glial responses to acute and chronic damage stimuli are very distinct.

Acute neuroinflammation

In response to acute injury, microglia change their resting ramified morphology to become ameboid and proliferate (Kettenmann *et al.*, 2011). Activation also increases microglial phagocytic potential, production of reactive oxygen and nitrogen species (ROS/RNS) and a panel of proinflammatory factors such as cytokines TNF and interleukin 1β (IL-1β), chemokines and surface receptors (CD68) (Fig. 2B). Such proinflammatory polarization has commonly been described as classical or M1 phenotype, as first observed for macrophages *in vitro*, although most researchers nowadays avoid this term due to the fact that the *in vivo* polarization in neurodegenerative diseases is never so clear-cut. Productive proinflammatory responses clear cellular debris leading to resolution of the injury, but also trigger a reactive anti-inflammatory response, which in turn suppresses inflammation and promotes repair (Fig. 2C). Microglial neurotrophic and anti-inflammatory factors include insulin-like growth factor 1 (IGF-1), brain-derived neurotrophic factor (BDNF), IL-10, IL-4, arginase 1, and scavenger receptors CD163 and chitinase-like protein 3 (YM1) (Philips and Robberecht, 2011). Anti-inflammatory microglia are also known as alternatively activated or M2 microglia to be distinguished from the proinflammatory M1 microglia in the early phase of an acute inflammatory response.

Chronic neuroinflammation in ALS

In contrast to limited, acute and resolving inflammatory processes, the progressive nature of CNS damage in ALS precludes complete damage control and does not follow a simple proinflammatory/M1 to anti-inflammatory/M2 transition (Chiu *et al.*, 2013). The initial acute phase is difficult to detect, suggesting that the primary damage is subtle and/or that the proinflammatory response seamlessly segues into an anti-inflammatory response (Fig 3A). Due to the difficulties of studying presymptomatic ALS in patients, most of the evidence on presymptomatic microglia comes from mouse models. Presymptomatic microglia in hSOD1 ALS mouse model express anti-inflammatory factors such as BDNF, IGF-1, IL-10, arginase 1, CD163 and YM1 (Appel *et al.*, 2011; Chiu *et al.*, 2013; Gravel *et al.*, 2016). They also have an attenuated response to subsequent inflammatory insults, which is at least in part due to decreased TLR2 expression and activity (Gravel *et al.*, 2016). The presymptomatic state is primarily maintained by secretion of IL-10 through autocrine signalling. An experimental increase in IL-10 production by microglia delays ALS onset slows progression and extends survival in the hSOD1 ALS mouse model. Furthermore, motoneurons in the presymptomatic stage are capable of providing negative co-stimulation via CX3CL1, which gets greatly reduced at disease onset (Zhang *et al.*, 2018). For these reasons, presymptomatic microglia are thought to have a neuroprotective role.

The transcriptional profiles of chronically activated microglia markedly change over the course of ALS. With the onset of symptoms, microglia diminish their expression of anti-inflammatory and growth factors, and increase proinflammatory factor production, such as NADPH oxidase 2, and proinflammatory cytokines IL-1β, TNF and IL-6 (Fig. 3B) (Zhao *et al.*, 2010; Beers *et al.*, 2011; Liao *et al.*, 2012). Notably, microglia in the most affected lumbar spinal cord regions are less responsive to inflammatory stimuli than healthy microglia, perhaps demonstrating exhaustion and/or desensitization (Nikodemova *et al.*, 2014). A similar microglial phenotype has also been reported in Alzheimer's disease and in models of experimental autoimmune encephalomyelitis, and was hence named disease-associated microglia (DAM) (Weissmann *et al.*, 2016; Keren-Shaul *et al.*, 2017; Krasemann *et al.*, 2017; Kang *et al.*, 2018; Deczkowska *et al.*, 2018*a*, *b*). This phenotype is achieved through the TREM2 signalling pathway

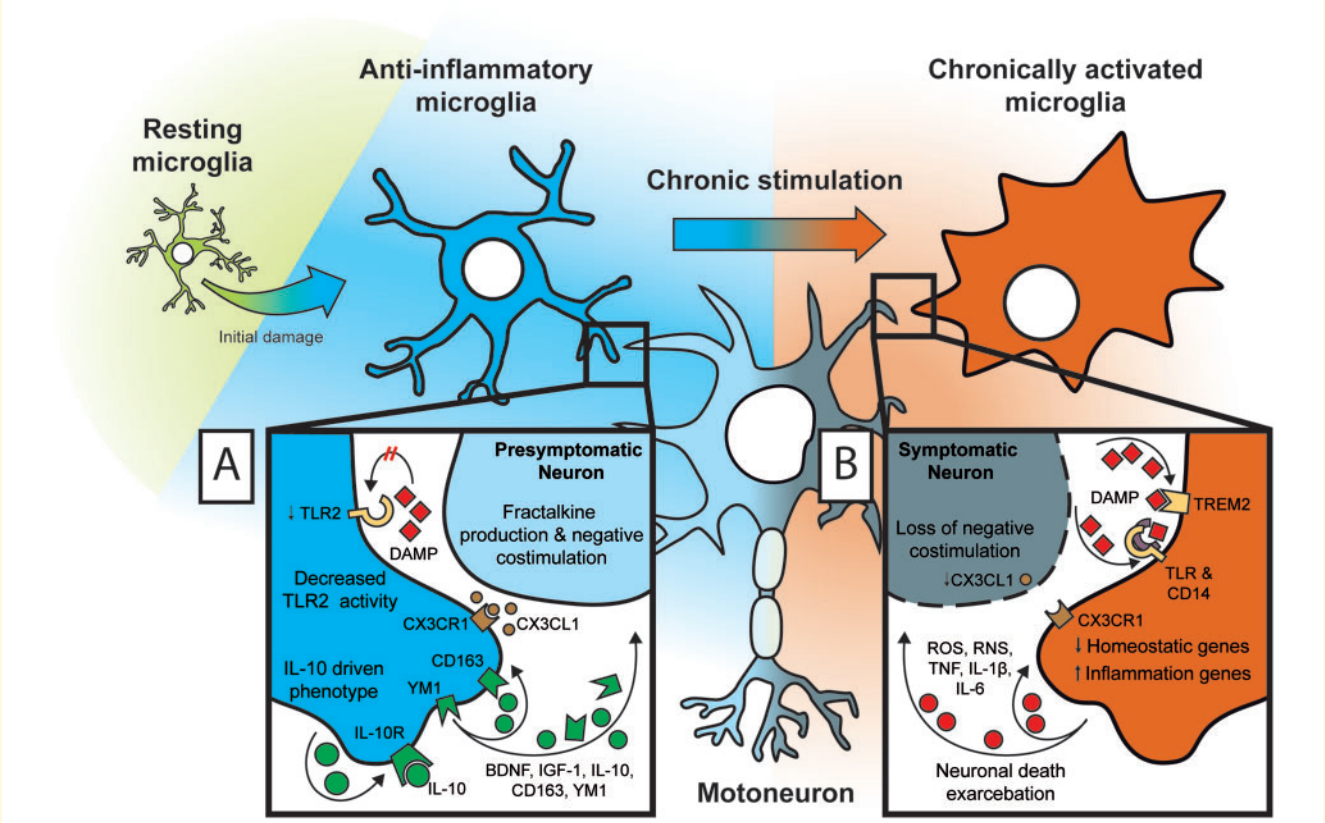


Figure 3 Chronic neuroinflammation. **(A)** The initial prolonged or repetitive damage in presymptomatic microglia elicits an anti-inflammatory phenotype. Neuronal negative co-stimulation with CD200 molecule and CX3CL1 is still active, and microglia show blunted proinflammatory response and neuroprotective properties: decreased TLR activity, increased production of anti-inflammatory cytokine IL-10, neurotrophic factors (BDNF, IGF-1) and scavenger receptors YM-1 and CD163. **(B)** Upon long-term chronic stimulation and/or repetitive hits microglia switch to highly proinflammatory state. Neurons in the symptomatic phase lose their ability to restrain microglial activation by negative co-stimulation, and various DAMPs (protein aggregates, debris, etc.) bind to their respective receptors (TLRs, TREM2, etc.) to activate downstream proinflammatory cascade, which results in profound changes in microglial transcriptional profiles (homeostatic genes are downregulated; proinflammatory genes are upregulated). Finally, proinflammatory microglia induce collateral neuronal damage, creating a vicious cycle that further amplifies inflammation.

following chronic stimulation by neuronal debris, protein aggregates, etc. The DAM phenotype is characterized by the downregulation of homeostatic genes such as *CX3CR1*, *P2RY12*, *TMEM119* and upregulation of *APOE*, *CCL3*, *CLEC7A*, *CST7*, *CTSE*, *GPNMB*, *ITGAX*, *LGALS3*, *LILRB4* or *LPL*, which are linked to microglial activity, phagocytosis and inflammation (Keren-Shaul *et al.*, 2017). The phagocytic DAM phenotype could be beneficial in plaque clearance in Alzheimer's disease, whereas its role in ALS is less clear. Notably, DAM microglia are preferentially enriched around amyloid β plaques in Alzheimer's disease, whereas their location in ALS was not reported. Since DAM do not express classic proinflammatory or anti-inflammatory markers and exhibit profound dysregulation of homeostatic genes (Keren-Shaul *et al.*, 2017; Deczkowska *et al.*, 2018*b*; Shi and Holtzman, 2018), they are distinct from M1 and M2 microglia. Of note, although DAM is a rather recent term, similar observations that microglia in ALS do not exhibit distinct textbook M1/M2 phenotypes have been reported previously (Chiu *et al.*, 2013; Butovsky *et al.*, 2014; Nikodemova *et al.*, 2014). Therefore, symptomatic ALS microglia cannot be phenotypically categorized as classical proinflammatory or 'M1', which is associated with acute and not chronic inflammation. This paradigm shift in nomenclature is necessary to properly represent pathogenic microglia in ALS, which is crucial for development of targeted therapies.

With so many differentially expressed factors in chronically activated microglia, distinguishing the cause from the effect has been notoriously difficult, and the initial triggers for dysbalanced inflammatory reaction in ALS are still unclear. One plausible hypothesis is that because healthy microglia exhibit blunted inflammatory responses in comparison to macrophages, they are less effective in healing and repairing, and more prone to sustain chronic inflammation (Fig. 3B). Several reports have linked chronic inflammation in ALS to TGF-β (Butovsky *et al.*, 2014; Cohen *et al.*, 2014; Endo *et al.*, 2015). TGF-β is mainly

produced by astrocytes, and regulates several opposing immune functions such as promotion of anti-inflammatory functions and regulatory T cells (Treg), or supporting proinflammatory Th17 T cells and fibrosis, depending on the surrounding microenvironment and cytokine milieu (Katsuno *et al.*, 2011; Butovsky *et al.*, 2014; Endo *et al.*, 2015). Activated microglia in mutant hSOD1 trigger differentiation of TGF-β-producing reactive astrocytes, suppress microglial neuroprotective functions and accelerate disease progression, whereas pharmacological blockade of TGF-β signalling extends survival (Iłzecka *et al.*, 2002; Endo *et al.*, 2015). Therefore, although TGF-β and perhaps other anti-inflammatory cytokines are initially beneficial, they are noxious on the long term (Cohen *et al.*, 2014).

Chronic stimulation shuts down glial neuroprotective functions and elicits the production of a variety of proinflammatory factors, making late-stage microglia and astrocytes from mutant hSOD1 mice toxic to motoneurons (Haidet-Phillips *et al.*, 2011; Liao *et al.*, 2012; Re *et al.*, 2014). Once generated, reactive astrocytes are sufficient to perpetuate the inflammatory reaction and/or neurotoxicity by themselves. This is partly explained by glutamate-mediated excitotoxic neuronal death due to lower levels of astrocytic excitatory amino acid transporter 2 (EAAT2) (Han and Whelan, 2010). Excitotoxicity is enhanced by proinflammatory cytokines such as TNF and motoneurons are exceptionally vulnerable to elevated glutamate levels. Aberrant populations of toxic microglia that express astrocytic markers and astrocytes expressing microglial markers have also been reported in ALS mouse models and patient samples (Díaz-Amarilla *et al.*, 2011; Komine *et al.*, 2018). Aberrant astrocytes develop at the onset of paralysis from $Iba1^{+}$ microglia, express astrocytic markers such as GFAP, S100β and connexin 43, are detected around the dying motoneurons in a rat mutant hSOD1 ALS model, and increase in number with disease progression (Trias *et al.*, 2013). The transdifferentiation processes leading to such aberrant cellular phenotypes are still unclear, but seem to be attractive therapeutic targets. Overall, ALS models demonstrate that a prolonged initial anti-inflammatory response is replaced by an excessive proinflammatory response towards the terminal stages of the disease, cautioning that therapeutic approaches will have to be stage-specific.

CNS macrophage and peripheral immune cell contribution to ALS pathogenesis

Growing evidence suggests that, in addition to microglia, several other innate immune cell subsets including macrophages, monocytes, dendritic cells (DC), natural killer (NK) cells, mastocytes and neutrophils are implicated in the ALS pathogenesis. Adaptive immune cells, in particular T cells play a key role as well, whereas the role of B cells and/or antibodies remains to be clarified. All these immune cell subsets likely exert a part or even most of their actions in the periphery as their access to the CNS is limited. Since ALS is a systemic disorder, regardless of their site of action, peripheral innate immune cells stand out as principal regulators of ALS pathogenesis.

Innate immunity

Similar to microglia, most CNS macrophages are tissue-resident long-lived cells derived from embryonic erythromyeloid precursors, with the exception of the choroid plexus macrophages, which partly originate from and are replenished by blood-derived monocytes (Prinz and Priller, 2014; Goldmann *et al.*, 2016; Prinz *et al.*, 2017). Their transcriptional profiles are more similar to microglia than to peripheral macrophages. We must bear in mind that to date it is still extremely challenging to distinguish the contribution of distinct myeloid cell types because of a substantial overlap in their activation markers. The same applies to DC and monocytes, which normally do not reside within the CNS, and their infiltration in ALS is still debated (Geissmann *et al.*, 2008; Mrdjen *et al.*, 2018). Some of the recent advances including transcriptional profiling, identification of a microglia-specific marker (transmembrane protein 119, Tmem119) and a potential monocyte marker not expressed on microglia (CD169), may help to distinguish innate immune cell types, but these approaches have not yet been validated and systematically implemented (Bennett *et al.*, 2016; Zondler *et al.*, 2016). Precise characterization of myeloid subsets will be crucial for understanding the extent of peripheral cell infiltration in ALS.

Several studies reported an increase in the monocyte chemoattractant CCL2 in the CNS and detected monocyte infiltration into spinal cords, suggesting that they promote disease progression and correlate to neuronal death in mutant hSOD1 ALS models (Henkel *et al.*, 2004; Mantovani *et al.*, 2009; Butovsky *et al.*, 2012). This was not corroborated in several other studies, which claimed that monocyte infiltration is beneficial. For example, chimeric mice lacking the CCL2 receptor CCR2 in the bone marrow succumbed to ALS faster than the mice harbouring WT bone marrow, suggesting that monocyte presence and/or recruitment is protective (Beers *et al.*, 2008). Similarly, experimentally induced increase in monocyte infiltration positively correlated with neuronal survival (Zondler *et al.*, 2016). In contrast, parabiosis, single-cell mass spectrometry and other experiments have demonstrated that peripheral monocytes minimally infiltrate the CNS in ALS mouse models (Solomon *et al.*, 2006; Ajami *et al.*, 2007, 2018). The latter notion has been further supported by RNA sequencing of acutely isolated spinal cord immune cells/microglia from ALS models, which revealed that

microglia do not share the molecular profiles of infiltrating monocytes, suggesting that without compromising BBB the infiltration of monocytes into CNS is limited (Chiu *et al.*, 2013; Ajami *et al.*, 2018). Taken together, it is still difficult to analyse the exact pattern and extent of monocyte infiltration in the brain and spinal cord in ALS models and patients. It is also notable that growing evidence suggests that endothelial cells are also affected in this disease, which in turn may cause progressive BBB alterations leading to infiltration of peripheral blood monocytes/macrophages. This has been confirmed in recent work by Garbuzova-Davis *et al.* (2017, 2019) as restoration of capillary integrity by delivery of human bone marrow stem cells reduced BBB permeability and delayed progression of disease in SOD1 mutant mice. Although the role and contribution of peripheral monocytes/macrophages in ALS-induced neurodegeneration remains elusive, these cells do have an important role in regulation of peripheral immunity. It is noteworthy that deregulation in peripheral blood monocyte immune profiles and function have been observed in sporadic ALS patients and may have some diagnostic and/or prognostic values. The role of monocytes in the regulation of peripheral immunity in ALS has been discussed in more details in the clinical chapter below, and some specific features of other peripheral innate subsets during ALS progression are listed in Box 1.

Adaptive immunity

T cells are present in negligible numbers in the CNS parenchyma, whereas slightly higher numbers are present in the meninges and the choroid plexus, with some entering the CSF through choroid plexus (Engelhardt and Ransohoff, 2005; Schwartz and Deczkowska, 2016). T cell infiltration to the CNS has for a long time been linked only to severe pathology, but it is now evident that CD4 T cells also exert important neuroprotective functions by improving cognition, and mediating CNS repair and recovery upon injury (Moalem *et al.*, 1999; Hauben *et al.*, 2001; Kipnis *et al.*, 2012). The presence of CD4 and CD8 T cells in the ventral horns and CD4 T cells in the corticospinal tracts has been reported in ALS patient autopsies around the1990s, but it was not immediately clear if they were harmful or beneficial (Troost *et al.*, 1989; Engelhardt *et al.*, 1993). The beneficial effects of CD4 T cells, in particular Tregs, are now widely accepted (listed in Box 2). In contrast to T cells, the relevance of B cells during ALS is poorly understood, although immunoglobulins G (IgG) reportedly accumulate in motoneurons (Box 2) (Engelhardt and Appel, 1990). Both antigen-specific and Fc-mediated effects have been reported. Therefore, Bcell responses in ALS patients remain an interesting subject and their exact role remains to be defined.

Box 1 Innate immunity in ALS pathogenesis

Monocytes

- The evidence on monocyte infiltration in ALS lesions is conflicting (Ajami *et al.*, 2007; Butovsky *et al.*, 2012; Zondler *et al.*, 2016).
- Familial and sporadic ALS patients have higher ratio of classical monocytes ($CD14^{++}CD16^{-}$) over non-classical monocytes ($CD14^{++}CD16^{++}$), which precedes disease onset; patients' monocytes possess decreased velocity, increased adhesion and impaired phagocytic activity (Zondler *et al.*, 2016).
- ALS patient monocytes have a proinflammatory phenotype (IL-1β, IL-8, FosB, CXCL1 and CXCL2 mRNA upregulation) that correlates with disease progression (Zhang *et al.*, 2005; Zhao *et al.*, 2017).

Macrophages

- CNS macrophages, found in the perivascular spaces of the BBB, meninges and choroid plexus, likely have overlapping roles with microglia in ALS pathogenesis (Prinz and Priller, 2014; Goldmann *et al.*, 2016; Prinz *et al.*, 2017).
- Macrophage infiltration and complement secretion in peripheral nerves increase with disease progression (Chiu *et al.*, 2009; Graber *et al.*, 2010; Lincecum *et al.*, 2010; Kano *et al.*, 2012).

NK cells

- NK cells increase with ALS progression in patients (Murdock *et al.*, 2017).
- NK cells infiltrate the motor cortex and the spinal cord of ALS patients and mouse models; NK cells kill motoneurons via NK2GD, reduce Treg infiltration and mediate microglial proinflammatory polarization via IFN-γ (Garofalo *et al.*, 2020).

Dendritic cells

- DC are reduced and dysfunctional in ALS patients' blood (Rusconi *et al.*, 2017).
- DC increase in ALS patients' spinal cords (Henkel *et al.*, 2004; Sta *et al.*, 2011).

Mast cells and neutrophils

- Masts cells infiltrate skeletal muscles at the neuromuscular junction and degranulate to help recruit neutrophils; neutrophils phagocytose degenerating axons and prevent reinnervation (Trias *et al.*, 2018).
- The drug masitinib targets these cells, reduces their infiltration and slows ALS progression in patients (Mora *et al.*, 2019).

The correlation of immune system activation to clinical phenotypes

Peripheral blood leukocytes are easily accessible and have been considered as possible ALS biomarkers. Decreased

Box 2. Adaptive immunity in ALS pathogenesis

T cells

- ALS patients have lower Treg numbers and function (Mantovani *et al.*, 2009; Henkel *et al.*, 2013; Beers *et al.*, 2017; Sheean *et al.*, 2018); at late disease stage, functional T cell defects and decreased numbers are seen in the peripheral immune compartments (Banerjee *et al.*, 2008).
- T cells in ALS patients and mouse models show proinflammatory Th1 and Th17 skewing at the late stages (Beers *et al.*, 2011; Henkel *et al.*, 2013; Saresella *et al.*, 2013).
- CD8 T cells specifically kill motoneurons in co-cultures, but CD8 T cell-deficient hSOD1 mice do not have shorter survival (Coque *et al.*, 2019); CD8 T cells positively correlate to neuronal function in the peripheral nerves (Nardo *et al.*, 2016).
- Proposed CD4 T cell neuroprotective mechanisms include: (i) Tregs and/or Th2 cells suppress microglial and effector T cell activation; (ii) Tregs and/or Th2 cells support recruitment of peripheral monocytes to the CNS; (iii) autoimmune CD4 T cells release the blockade of the immune cell gateway to the CNS through the choroid plexus (Zhao *et al.*, 2012; Ueno *et al.*, 2013; Baruch *et al.*, 2014; Kunis *et al.*, 2015).

B cells and antibodies

- B cells have no impact on disease progression in a mouse mutant hSOD1 model (Naor *et al.*, 2009).
- IgGs accumulate in motoneurons; IgG from ALS patients enter and stimulate motoneurons, astrocytes and microglia (Engelhardt and Appel, 1990; Appel *et al.*, 1991; Pagani *et al.*, 2011; Bataveljic *et al.*, 2014; Miloševic *et al.*, 2017).
- Some antibodies recognize aggregated proteins and neurofilaments, but their pathogenicity is unclear (May *et al.*, 2014; Malaspina *et al.*, 2015).
- Passive antibody transfer from ALS patients into mice increases serum and spinal cord cytokines (TNF, IL-6 and IL-10) and elicits motoneuron degeneration (Pullen *et al.*, 2004; Obál *et al.*, 2016).

counts of CD4 T cells and increased counts of total leukocytes, neutrophils, $CD16^{+}$ and $CD16^{-}$ monocytes and NK cells have been reported in ALS patients (Murdock *et al.*, 2017). Importantly, the early changes in leukocyte numbers, in particular neutrophils and CD4 T cells, significantly correlated with disease progression measured by Amyotrophic Lateral Sclerosis Functional Rating Scale – Revised (ALSFRS-R) functional score. T cell data were corroborated in several other studies. For example, Treg numbers and function negatively correlate to disease severity i.e. low numbers are predictive for rapid disease progression and short survival (Fig. 4) (Henkel *et al.*, 2013; Sheean *et al.*, 2018), whereas there is moderate negative correlation between Th1 and Th17 T cells, ALSFRS-R and forced vital capacity (Jin *et al.*, 2020). Another study observed a positive correlation between high baseline neutrophil-to-lymphocyte ratio and shorter survival, proposing that it can reflect the degree of neuroinflammation in ALS patients (Choi *et al.*, 2020). A higher ratio of classical monocytes ($CD14^{++}CD16^{-}$) over non-classical monocytes ($CD14^{++}CD16^{++}$) is found in familial and sporadic ALS patients and precedes disease onset (Zondler *et al.*, 2016). Other insights into ALS pathogenesis were provided by gene profiling studies. Zhang *et al.* showed that peripheral blood monocytes isolated from sporadic ALS patients strongly upregulate a cluster of LPS-TLR4 associated genes including those encoding for IL-1β, IL-8, FosB, CXCL1 and CXCL2, and that their level of activation correlates with disease severity (Zhang *et al.*, 2005; Zhao *et al.*, 2017). Surprisingly, they found that this upregulation correlated with elevated LPS plasma levels in sporadic ALS patients, suggesting that the molecular mechanisms of disease-associated deregulation of peripheral immunity may be more complex than initially thought. Importantly, immune profiles of peripheral monocytes at the mRNA level differ in rapidly and slowly progressing ALS patients, with rapidly progressing patients exhibiting more proinflammatory phenotypes (Zhao *et al.*, 2017). In a recent study, TGF-β1 and TGF-β3 levels were also recently reported as potential markers of disease ALS severity, showing a significant negative correlation with ALSFRS-R (Duque *et al.*, 2020). Therefore, the level of peripheral inflammation may predict disease progression.

Some immune biomarkers associated with ALS have also been discovered in the CSF. For example, an increase in the level of macrophage-derived chitinases (CHIT1, CHI3L1 and CHI3L2), a signal of microglia/macrophage activation, was recently observed in ALS patients, and chitinase levels correlated with disease progression, severity, survival and other neurodegeneration markers such as phosphorylated neurofilament heavy chain (Steinacker *et al.*, 2018; Thompson *et al.*, 2018; Gille *et al.*, 2019; Vu *et al.*, 2020). A more detailed analysis of inflammatory factors, in both serum and CSF, and their correlation to disease severity is provided in Table 1.

It is notable that only few studies correlated immune parameters to specific ALS gene mutations. A study by Olesen *et al.* (2020) described differences in immune parameters and survival times in ALS subtypes. In particular, patients with sporadic ALS had higher CSF TNF compared with those with *C9orf72* mutations, whereas patients with *C9orf72* mutations had higher CSF IFN-α compared to those with *SOD1* mutations or with sporadic ALS. The survival was negatively correlated with plasma IL-10, TNF and TNF-related apoptosis-inducing ligand (TRAIL), while in *C9orf72* patients the levels of plasma IL-1β correlated negatively with survival. In addition, CHIT1 concentration was markedly increased in the CSF of symptomatic familial ALS patients, including *C9orf72*, *SOD1*, *TARDBP* and *FUS* mutation carriers,

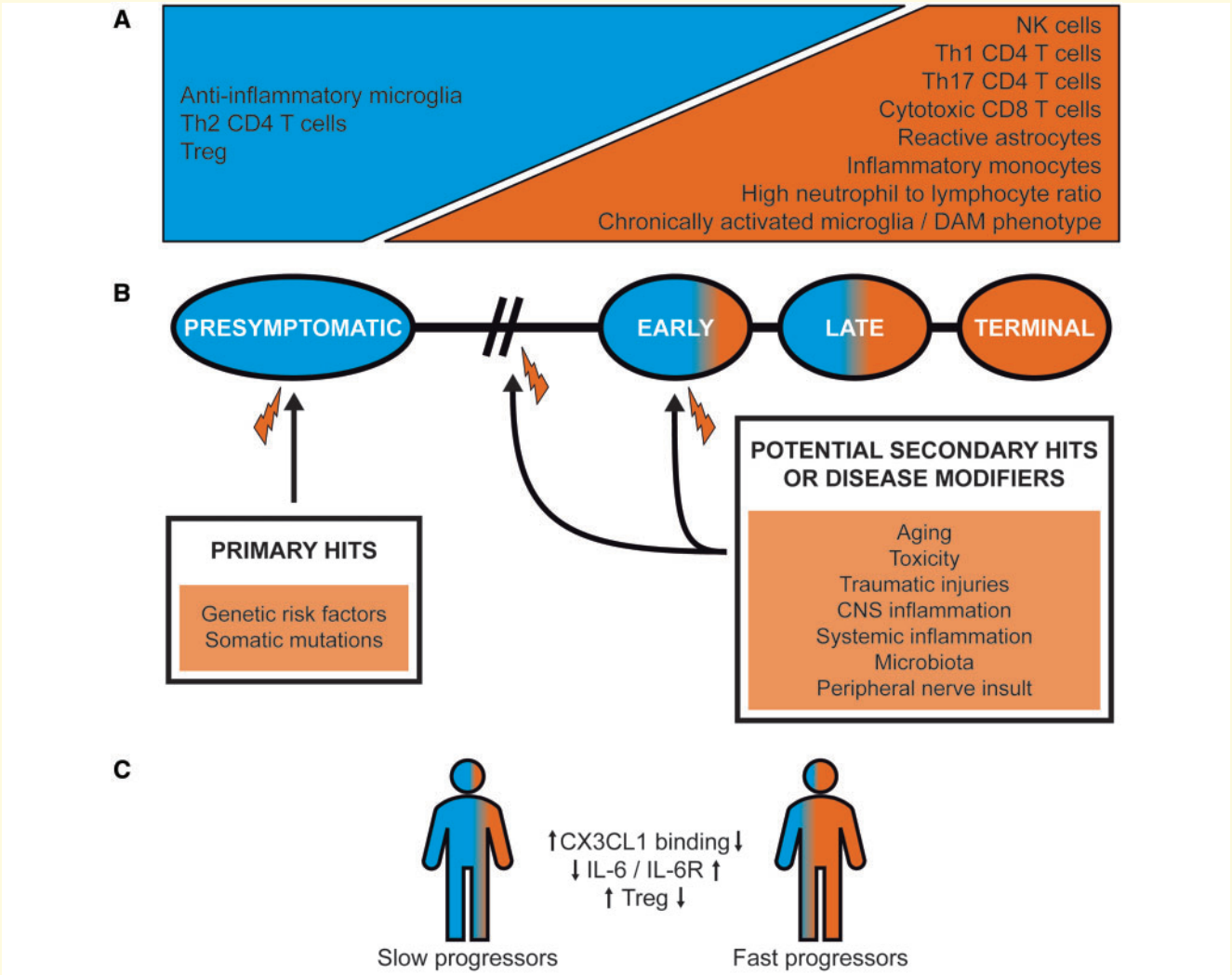


Figure 4 The timeline of ALS progression. **(A)** The progressive loss of neuroprotective immune responses over the course of ALS is depicted. **(B)** The potential prolonged and/or repetitive hits that affect ALS pathogenesis are shown. Inherited or sporadic mutations in ALS-associated genes could serve as primary hits that negatively affect to the initial anti-inflammatory phase, whereas secondary hit/s would set off the rapidly progressive stage marked with uncontrolled inflammation. **(C)** The disease progression is shaped by the immune factors. Various polymorphisms or other factors that result in lower-binding capacity of CX3CL1, higher levels of IL-6 and IL-6R, or reduced Treg numbers and function, are linked to rapid disease progression.

when compared to both healthy controls or asymptomatic mutation carriers (Oeckl *et al.*, 2019). CHIT1 levels strongly correlated with axonal degeneration markers, suggesting that neuroinflammation is linked to the symptomatic phase of disease. Moreover, in a cohort of *C9orf72* patients, a different protein profile, including neurofilament medium polypeptide (NEFM) and CHIT1, was observed in ALS and frontotemporal dementia patients, suggesting as these markers could distinguish between these two phenotypes (Barschke *et al.*, 2020). Further research is needed to understand exactly which immune mediators are relevant for disease pathogenesis, and pinpoint other genetic variants with distinct immunological patterns.

How does immunity turn from friend to foe during ALS?

We have listed various immune cell subsets and neuroprotective and neurotoxic immune mechanisms that contribute to ALS pathogenesis. Extensive *in vitro* and *in vivo* evidence shows that inflammation can be secondary to prolonged and/or repetitive neuronal damage so this will be discussed first. After that, we will detail recent findings on immune system dysregulation as a potential initial trigger and/or a key disease modifier. This is in accordance with the recent mapping of

Table 1 Relevant immunological mediators and their correlation with clinical disease course

Biomarker	Biofluid	Significance	References
TGF-β1	Blood	Higher levels with disease duration Negative correlation with ALSFRS-R	Houi *et al.* (2002) and Duque *et al.* (2020)
TGF-β3	Blood	Negative correlation with ALSFRS-R	Duque *et al.* (2020)
IL-2	Blood	Decrement with disease duration Poor survival	Ehrhart *et al.* (2015) and Lu *et al.* (2016)
IL-4	CSF	Slower disease progression; prevalent lower motor neuron phenotype	Furukawa *et al.* (2015)
IL-5	Blood	Positive correlation with CK	Lu *et al.* (2016)
IL-6	Blood CSF and blood	Positive correlation with CRP and end-stage disease Increment with respiratory dysfunction	Moreau *et al.* (2005) and Lu *et al.* (2016)
IL-8	Blood	Increment with disease duration	Ehrhart *et al.* (2015)
IL-10	Blood CSF	Negative correlation with survival Slower disease progression; prevalent lower motor neuron phenotype	Furukawa *et al.* (2015) and Olesen *et al.* (2020)
IL-13	Blood	Negative correlation with ALSFRS-R and disease progression rate	Shi *et al.* (2007)
TNF	Blood	Positive correlation with NfL Increment with respiratory dysfunction Negative correlation with survival	Moreau *et al.* (2005), Lu *et al.* (2016) and Olesen *et al.* (2020)
Ferritin	Blood	Poor survival	Lu *et al.* (2016)
IFN-γ	Blood CSF	Increment with disease duration and progression rate Increment with disease duration and progression rate Shorter survival	Liu *et al.* (2015) and Guo *et al.* (2017)
bFGF	CSF	Slower disease progression and longer disease duration	Guo *et al.* (2017)
VEGF	CSF	Slower disease progression and longer disease duration	Guo *et al.* (2017)
MIP-1α	CSF	Slower disease progression and longer disease duration	Guo *et al.* (2017)
CCL2	CSF	Worse disease severity and faster progression	Guo *et al.* (2017)
TRAIL	Blood	Negative correlation with survival	Olesen *et al.* (2020)
CHIT1	CSF	Positive correlation with rate of disease progression and level of NfL and NfH; negatively correlated with survival	Steinacker *et al.* (2018), Thompson *et al.* (2018), Gille *et al.* (2019), and Vu *et al.* (2020)
CHI3L1 (YKL-40) CHI3L2	CSF	Positive correlation with rate of disease progression and level of NfH	Thompson *et al.* (2018) and Vu *et al.* (2020)

ALSFRS-R = Amyotrophic Lateral Sclerosis Functional Rating Scale – Revised; bFGF = basic fibroblast growth factor; CK = creatine kinase; CRP = C-reactive protein; MIP-1α = macrophage inflammatory protein-1 alpha; NfL = neurofilament-light chain; NfH = neurofilament-heavy chain; TRAIL = TNF-related apoptosis-inducing ligand; VEGF = vascular endothelial growth factor.

several important immune-related genes and cellular pathways in ALS. Interestingly, two seemingly opposite immune defects were linked to these mutations: excessive inflammation and ineffective immune responses. However, the line between these two conditions is blurred because some immunodeficient states manifest as hyperinflammation due to the inability to contain pathogens (or even commensal bacteria) and/or clear tissue damage (Marks *et al.*, 2010; Fodil *et al.*, 2016). These processes comprise newer concepts in the pathogenesis of ALS, whose roles have only recently begun to be clarified.

Neuroinflammation secondary to prolonged and/or repetitive neuronal damage

As discussed earlier, typical ALS DAMPs arise from neuronal debris, but even prior to neuronal death, aggregated TDP-43 or SOD1 (mutant and/or oxidized WT), can be secreted or exocytosed by various CNS cells, and directly trigger inflammation (Urushitani *et al.*, 2006; Ezzi *et al.*, 2007; Zhao *et al.*, 2010; Roberts *et al.*, 2013; Zhao *et al.*, 2015). The current model proposes that the predominant initial response of the immune system to aggregated proteins or similar stressors is neuroprotection, which at some still ill-defined point, when the damage can no longer be eliminated or contained, shifts to full-blown neuroinflammation and neurotoxicity, leading to an exacerbation of the primary damage (Fig. 4A). Data to support this neuroprotective to neurotoxic shift came largely from studies in mutant hSOD1 ALS models, in which the initial presymptomatic and early symptomatic phases are dominated by anti-inflammatory immune responses (anti-inflammatory microglia, Treg and Th2 CD4 T cells), whereas late symptomatic and terminal stages are dominated by proinflammatory immune responses (proinflammatory microglia and astrocytes, inflammatory monocytes, cytotoxic CD8 T cells, Th1 and

Th17 CD4 T cells, NK cells and neutrophils) (Henkel *et al.*, 2013; Zhao *et al.*, 2013; Murdock *et al.*, 2017; Sheean *et al.*, 2018; Choi *et al.*, 2020; Garofalo *et al.*, 2020; Jin *et al.*, 2020). As previously discussed, once that a hyperinflammatory state of microglia or astrocytes is established, these glial cells directly become toxic and can kill motoneurons (Roberts *et al.*, 2013; Re *et al.*, 2014; Zhao *et al.*, 2015).

Although the exact turning point in this process remains unclear, it is reasonable to hypothesize that microglia and other immune cell subsets become neurotoxic upon facing prolonged and/or repetitive hits (Fig. 4B). The environmental factors that lead to ALS are poorly defined, except for aging, which is a common predisposing factor for all neurodegenerative diseases (Lucin and Wyss-Coray, 2009). Aged microglia exhibit enhanced and prolonged proinflammatory response to LPS challenge, accompanied with fractalkine receptor (CX3CR1) downregulation, which makes them refractory to neuronal immunosuppressive cues, whereas the aged choroid plexus decreases leukocyte entry from the blood, which reduces its neuroprotective potential (Godbout *et al.*, 2005; Wynne *et al.*, 2010; Baruch *et al.*, 2014). Other environmental factors that may act as secondary hits or disease modifiers that could set off neurodegeneration include smoking, hormones, growth factors, head trauma, gut microbiome composition, peripheral nerve insults, and systemic and CNS inflammation (Nguyen *et al.*, 2004; Bilic *et al.*, 2006; Chen *et al.*, 2007; Erny *et al.*, 2015; Hancevic *et al.*, 2019; Schram *et al.*, 2019; Zhan and Fang, 2019).

Cytokines in systemic infections act as potential risk factors due to various mechanisms that allow microglia to 'mirror' systemic inflammation (Perry and Holmes, 2014). For this reason, chronic systemic LPS administration precipitates ALS symptoms in the mutant hSOD1 mouse model (Nguyen *et al.*, 2004). Viral infections have also been proposed as potential risk factors as several studies detected viral genome elements in ALS patients' spinal cords, mainly belonging to enteroviruses (Berger *et al.*, 2000; Giraud *et al.*, 2001; Xue *et al.*, 2018*a*; Castanedo-Vazquez *et al.*, 2019). Furthermore, a small fraction of patients infected with human immunodeficiency virus-1 (HIV-1) or human T cell leukaemia virus-1 (HTLV-1) progress to an ALS-like syndrome, which responds to antiretroviral therapy (Alfahad and Nath, 2013). ALS patients were reported to have significantly higher expression of human endogenous retroviral sequences in motoneurons compared to control subjects, and mice overexpressing endogenous retrovirus K develop an ALS-like syndrome (Douville *et al.*, 2011; Li *et al.*, 2015). Endogenous retrovirus K reactivation was found to be driven by neuroinflammatory cytokines TNF and IFN-γ, which act through IFN-stimulated responsive elements and NF-κB-binding sites in the viral promoter (Manghera *et al.*, 2016). This allows for the possibility of a vicious cycle in which proinflammatory cytokines produced by microglia reactivate endogenous retrovirus K, which in turn supports chronic microglial activation and predisposes patients to ALS.

The gut microbiome is an important source of bioactive metabolites, and its imbalance can contribute to various diseases (Amedei and Boem, 2018; Di Gioia *et al.*, 2020). It represents an important interface between the environment and the immune system, capable of affecting the CNS through microbial toxins or fermentation products, which can in turn trigger protein aggregation, proinflammatory cytokine secretion and microglial activation, among others (Erny *et al.*, 2015). The link between gut microbiome and ALS is nowadays well-established in several animal models. SOD1 mice exhibit reduced colonic tight junction proteins and increased intestinal permeability prior to disease onset, and the levels and composition of commensal bacteria correlate with disease severity and exacerbation of the symptoms (Fang, 2016; Zhang *et al.*, 2017; Blacher *et al.*, 2019). Further confirmation came from *C9orf72*$^{-/-}$ mice in which reduced abundance of immune system-stimulating bacteria protected the mice from premature mortality and significantly attenuated their underlying systemic inflammation (Burberry *et al.*, 2020). These findings suggest that in *C9orf72*$^{-/-}$ mice microbiome composition may be an important disease modifier for both onset and progression. Several studies have also advanced from preclinical to patients. Notably, a significant difference is observed in the global bacterial gene content in ALS patients compared with healthy controls (Rowin *et al.*, 2017; Brenner *et al.*, 2018; Blacher *et al.*, 2019; Di Gioia *et al.*, 2020). The alteration of gut microbiome and the other mechanisms listed in this section (prolonged hits from degenerating neurons, environmental factors, CNS trauma, systemic inflammation, etc.) could explain how initially protective microglial functions can, at a certain point, break down and lead to hyperinflammation, collateral neuronal damage and unrelenting ALS progression.

Excessive inflammation as a potential ALS trigger and/or disease modifier

A rare proof of principle that hyperactivated microglia can precede neuronal damage came from an experimental mouse model harbouring constitutively active IκB kinase β (IKKβ) in microglia, which led to a chronic NF-κB activation and gliosis, resulting in motoneuron death and a neurodegeneration similar to ALS (Frakes *et al.*, 2014). Conversely, in a mutant hSOD1 model selective downregulation of NF-κB in microglia, but not in astrocytes, considerably prolonged the life span. However, immune system activation is complex and harmful processes in some cells or at some disease stages, can be beneficial in others. For example, astrocyte-specific NF-κB activation delayed the progressive phase of the disease by

facilitating microglial transition to an anti-inflammatory state, enhancing autophagy and decreasing aggregated mutant hSOD1 (Ouali Alami *et al.*, 2018). However, at later stages, it favoured a proinflammatory state of microglia and accelerated disease progression. These experiments opened the possibility that excessive inflammation through NF-κB pathway activation could be a primary pathogenic step in ALS.

The first ALS patient mutations proposed to be linked to excessive inflammation were reported in the *OPTN* gene encoding for an ubiquitin-binding adaptor protein optineurin (Maruyama *et al.*, 2010). Due to its homology and competition to NF-κB essential modulator, optineurin was reported to act as a negative regulator of NF-κB signalling (Zhu *et al.*, 2007; Maruyama *et al.*, 2010; Akizuki *et al.*, 2013). Microglial NF-κB activation was detected in autopsies of a homozygous *OPTN* mutation carrier and sporadic ALS patients (Sako *et al.*, 2012). Unexpectedly though, extensive studies performed in various peripheral innate immune cell subsets and microglia from the $OPTN^{-/-}$ model or models lacking the ubiquitin-binding activity showed that optineurin is dispensable for NF-κB activation and production of proinflammatory cytokines (Gleason *et al.*, 2011; Munitic *et al.*, 2013; Pourcelot *et al.*, 2016; Slowicka *et al.*, 2016; Markovinovic *et al.*, 2018). One group initially reported that $OPTN^{-/-}$ mice had a slight increase in proinflammatory cytokines, dysmyelination and deficits in vertical rearing activity, but other studies reported no neuroinflammation, dysmyelination and/or neurological defects (Ito *et al.*, 2016; Slowicka *et al.*, 2016; Dermentzaki *et al.*, 2019). However, an ubiquitin-binding optineurin patient mutation (E478G) lentivirally delivered to the mouse motor cortex, triggered secretion of proinflammatory cytokines and neuronal death (Liu *et al.*, 2018). Therefore, although most studies dismissed excessive inflammation as a mechanism of action of *OPTN* mutations in ALS, additional functional studies will be necessary to understand if individual *OPTN* mutations such as E478G act by distinct pathogenic mechanisms. Of note, optineurin is a multifunctional protein that also regulates the IFN-β pathway and autophagy, which will be discussed in the next chapter.

An observation that the *C9orf72* gene has higher expression in microglia and other myeloid cells than in neurons, opened up the possibility that its loss- and/or gain-of-function may affect (neuro)inflammation (DeJesus-Hernandez *et al.*, 2011; Gendron *et al.*, 2013; O'Rourke, 2016). Gain of function of *C9orf72* gene is observed either as toxicity linked to formation of RNA foci or as generation of dipeptide protein repeats. $C9orf72^{-/-}$ mice show defective lysosomal functions and increased production of proinflammatory cytokines in myeloid cells, splenomegaly, lymphadenopathy and production of self-reactive antibodies, indicating the appearance of autoinflammation and/or autoimmunity (Atanasio *et al.*, 2016; O'Rourke, 2016). Due to the breadth of inflammatory markers expressed in these mice, we classified these mutations as potentially hyperinflammatory. It is notable though, that the initial trigger for hyperinflammation is unknown, and could in theory be defective lysosomal waste removal, which would in turn classify these mutations as an immunodeficiency, as discussed in the next chapter. This remains to be further investigated. However, neither whole body nor conditional *C9orf72* loss in neurons or glia cause motoneuron degeneration in a mouse model (Koppers *et al.*, 2015; O'Rourke, 2016). For this reason, it is unlikely that these models appropriately mimic human disease and that dysregulated inflammation is by itself sufficient to cause the disease in patients carrying *C9orf72* mutations. Dipeptide protein repeat-mediated toxicity has been linked to inflammation as well. Several studies of *C9orf72* BAC and poly-GA transgenic mouse models with widespread accumulation of dipeptide protein repeats showed appearance of either microgliosis and/or astrogliosis in the spinal cords and several brain regions (Zhang *et al.*, 2015; Liu *et al.*, 2016; Schludi *et al.*, 2017). However, certain discrepancies in the extent and breadth of glial activation were reported in these otherwise similar models, necessitating further clarification. Nevertheless, the crosstalk of loss of function to other proposed mechanisms of action of *C9orf72* mutations, which include toxicity of dipeptide repeats, RNA toxicity and impaired autophagy, could be necessary for disease appearance (Sellier *et al.*, 2016; Sullivan *et al.*, 2016).

A recent genome-wide association studies identified *TNIP1* as a potential risk locus in Chinese ALS patients. The *TNIP1* gene encodes for TNFAIP3-interacting protein 1, also known as A20-binding inhibitor of NF-κB (ABIN1) (Benyamin *et al.*, 2017). ABIN1 has an ubiquitin-binding region that is highly homologous to optineurin and can shut down the NF-κB pathway via recruiting a deubiquitinating enzyme A20. Furthermore, polymorphisms in cytokine and chemokine receptors have been recently discovered as disease modifiers in ALS patients (Fig. 4C). Carriers of *CX3CR1* 249I allele, which has lower CX3CL1 binding capacity, have a substantially faster disease progression (Lopez-Lopez *et al.*, 2014; Wosiski-Kuhn *et al.*, 2019*b*). Similarly, carriers of the IL-6R Asp358Ala variant have higher IL-6 and IL-6R in the sera and CSF (Wosiski-Kuhn *et al.*, 2019*b*). Notably, neither *CX3CR1* nor *IL-6R* polymorphisms increase the risk of disease, but both lead to a rapid disease progression and shorter survival. This argues that immune system dysregulation linked to these polymorphisms is insufficient to cause the disease by itself but instead works together with other neurotoxic hits. Whether this is necessary for all ALS mutations affecting the immune system is still unknown; proofs of principle from mouse studies demonstrate that hyperactivated microglia can exert toxicity by itself (Frakes *et al.*, 2014), but most of the other models discussed in this chapter were not cell-specific. Overall, several genes that directly regulate the

immune system have been characterized, demonstrating that excessive responses to damage could in these particular cases be a primary cause or key disease modifier in ALS.

Immunodeficiency as a primary ALS trigger

Immunodeficiencies, subdivided into defects of innate or adaptive immunity, comprise inabilities to mount appropriate immune responses required for damage elimination. They are commonly linked to impaired responses to infection and are rarely discussed in the context of neurodegeneration. Here we propose that inefficient inflammatory factor secretion and/or clearance of neurodegenerative DAMPs reported for some ALS-causing mutations represent an innate immunodeficiency. An early proof of principle that innate immunodeficiency can accelerate ALS came from a chimeric mouse hSOD1 model that was (upon irradiation) reconstituted with WT or myeloid differentiation factor 88 (Myd88)-deficient bone marrow, replacing endogenous microglia. Myd88 is an adaptor to all TLRs except TLR3 and activates the NF-κB and mitogen-activated protein kinases (MAPK) pathways. Compared to microglial replacement with WT cells, microglial replacement with Myd88-deficient cells was associated with earlier disease onset and decreased survival (Kang and Rivest, 2007). Another example came from an experimental TIR-domain-containing adapter-inducing IFN-β (TRIF)-deficient mutant hSOD1 model (Komine *et al.*, 2018). TRIF is an adaptor protein that primarily regulates IFN-β responses. It conveys signals from TLRs, but in contrast to MyD88, TRIF is activated via TLR3 or (together with MyD88) via TLR4, primarily resulting in TBK1 activation (O'Neill and Bowie, 2007). TBK1 is the primary kinase that phosphorylates transcription factor IFN regulatory factor 3 (IRF3) required for antiviral IFN-β production (Kielian, 2006). Whole-body TRIF deficiency substantially accelerated the development of ALS symptoms and shortened survival in the mutant hSOD1 model, whereas MyD88 deficiency had no effect (Komine *et al.*, 2018). This suggests that IFN-β regulated responses delay pathology in that model.

Recently discovered ALS-linked mutations in *TBK1* (Fig. 5) may also affect the IFN-β pathway and cause innate cell immunodeficiency (Maruyama *et al.*, 2010; Cirulli *et al.*, 2015; Freischmidt *et al.*, 2015). While the role of TBK1 in neurodegeneration is rather new, neuroprotective effects of IFN-β in multiple sclerosis are well-known. It promotes microglial phagocytosis, regulates the switch from pro- to anti-inflammatory microglia, etc. (Cohen *et al.*, 2014; Kocur *et al.*, 2015). IFN-β also promotes neurite branching and autophagy in neurons, thus protecting against α-synuclein pathology and motor and cognitive defects in mice (Ejlerskov *et al.*, 2015). In ALS patients bearing a *TBK1* mutation, it was suggested that the TBK1-induced pathology was caused by a loss of function from haploinsufficiency as TBK1 mRNA and protein levels were reduced in comparison to sporadic ALS patients (Freischmidt *et al.*, 2015; Pottier *et al.*, 2015). In mice, TBK1 deficiency is lethal, whereas haploinsufficiency is insufficient to cause the disease by itself (Brenner *et al.*, 2019; Gerbino *et al.*, 2020). However, in a mutant hSOD1 model, haploinsufficiency of TBK1 or a loss-of-function mutation in $TBK1^{R228H}$ showed a biphasic effect on the disease course: accelerating disease onset, but afterwards suppressing the appearance of neurotoxic microgliosis and astrogliosis, and extending survival. Primary microglia from $TBK1^{+/-}$ $SOD1^{G93A}$ mice exhibited diminished production of proinflammatory cytokines *in vitro* (Brenner *et al.*, 2019). $TBK1^{R228H}$ $SOD1^{G93A}$ mice led to diminished IRF3 activation and decreased induction of IFN-stimulated genes at the late stage of the disease. It was thus proposed that diminished inflammatory responses due to TBK1 insufficiency slow down the disease progression at late stages (Gerbino *et al.*, 2020). Given that TBK1 also has a role in promoting autophagy, the authors proposed that the accelerated early-stage disease in $TBK1^{+/-}$ mutant hSOD1 ALS model resulted from defective autophagy (Brenner *et al.*, 2019; Gerbino *et al.*, 2020). However, this does not exclude the possibility that innate immunodeficiency resulting from diminished IFN-β production could contribute as well. Additional complexity might arise from the fact that more than 70 mutations in *TBK1* have been found in ALS patients, which differently affect the kinase activity and may have different mechanisms of action (CirulLi *et al.*, 2015; Freischmidt *et al.*, 2015; Oakes *et al.*, 2017; Ye *et al.*, 2019).

Insufficiency of optineurin also leads to diminished IFN-β responses in microglia (Fig. 5) (Markovinovic *et al.*, 2018). Optineurin acts as an adaptor protein necessary for optimal TBK1 activation in various other innate immune cell subsets, suggesting that they likely act on the same axis in the pathogenesis of ALS (Munitic *et al.*, 2013; Meena *et al.*, 2016; Pourcelot *et al.*, 2016). Moreover, previous findings linked optineurin deficiency with diminished acute immune response and neutrophil recruitment to the site of infection in mice (Chew *et al.*, 2015; Smith *et al.*, 2015), hence opening a possibility that inadequate first response to damage due to disruption in the optineurin-TBK1 axis could trigger neurodegeneration. In addition to diminished inflammatory response, ALS-linked mutations in optineurin also lead to diminished autophagy and mitophagy (Heo *et al.*, 2015; Lazarou *et al.*, 2015; Richter *et al.*, 2016; Evans and Holzbaur, 2020). Interestingly, to become an autophagy adaptor, optineurin must be phosphorylated by TBK1. It is thus plausible that a breakdown in the crosstalk between autophagy and inflammation triggers disease (Markovinovic *et al.*, 2017). The impaired autophagy itself could also lead to an accumulation of aggregated proteins, build-up of ROS from damaged mitochondria, and diminished elimination of bacteria and viruses. If these events are coupled by inadequate inflammatory response that cannot contain the damage, the likely final

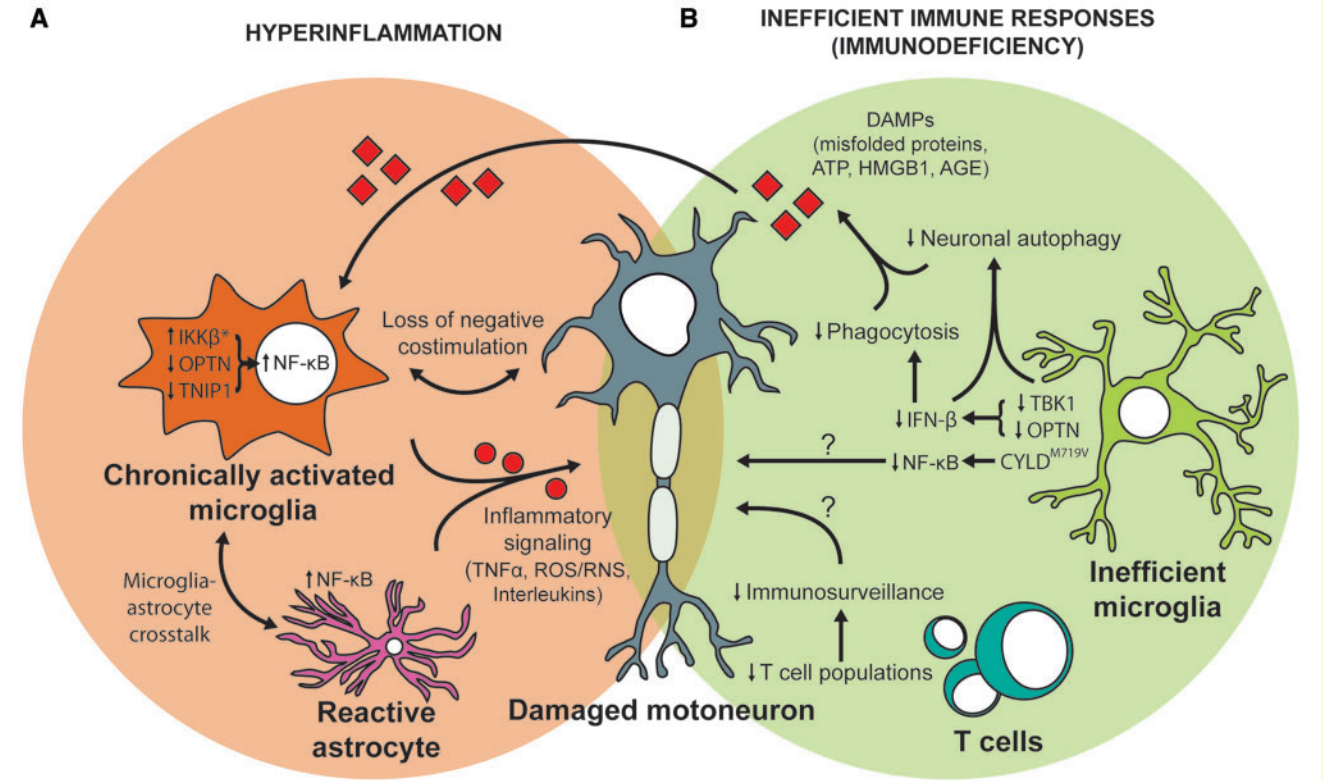


Figure 5 Hyperinflammation and immunodeficiency as primary triggers of motoneuron death in ALS. (A) Microglial skewing towards uncontrolled inflammation could be a repercussion of inadequate shut down of NF-κB signalling exerted by constitutive activation of IKKβ (*clinical correlate not yet reported), or mutations in proposed negative regulators *OPTN* and *TNIP1*. Overactivated NF-κB in reactive astrocytes elicits similar effects. Such excessive proinflammatory factor production from chronically activated glia ultimately causes motor neuron death. **(B)** The inability of microglia to optimally respond to damage is a potential trigger for neurodegeneration in ALS. ALS-linked loss-of-function mutations in *OPTN* and *TBK1* lead to decreased production of IFN-β, dysregulated inflammatory responses, reduced phagocytic capacity of microglia and decreased neuronal autophagy. ALS-linked mutant $CYLD^{M719V}$ was proposed to impair autophagy flux and reduce NF-kB activation. Such inadequate response to damage finally leads to accumulation of various DAMPs (protein aggregates, ATP, HMGB1, etc.), thus sparking neurotoxic chronic inflammation. Loss of immunosurveillance by the adaptive immunity, in particular T cells, could contribute to dysregulated responses and neurotoxicity.

outcome is accumulation of PAMPs/DAMPs, chronic inflammation and neuronal death (Fig. 5).

Newly discovered missense mutation in the gene encoding for cylindromatosis (CYLD) protein, a deubiqutinase for K63-linked polyubiquitin chains that terminates NF-κB and TBK1 signalling, was recently linked to ALS (Dobson-Stone *et al.*, 2020). ALS-linked mutant $CYLD^{M719V}$ exhibited increased deubiquitinase activity and subsequently reduced NF-κB activation in HEK293 cells (Fig. 5). The exact mechanism of action is still unclear since $CYLD^{M719V}$ also impairs autophagy by blocking autolysosome formation. This perhaps represents additional evidence that the combination of impaired autophagy and inadequate inflammatory response can promote motor neuron death, but further studies in primary cells and mouse models are needed to confirm that hypothesis.

Several lines of evidence have also suggested that dysfunction of adaptive immunity could contribute to ALS pathogenesis. Thymic involution and reduced T cell progenitor numbers are found in the mutant hSOD1 ALS mouse model, which results in reduced thymic output and restricted T cell repertoire in both mouse models and ALS patients (Fig. 5) (Seksenyan *et al.*, 2010). Mutant hSOD1 ALS mice also mount diminished T cell response to vaccination in comparison to WT mice (Banerjee *et al.*, 2008; Kunis *et al.*, 2015). Furthermore, immunodeficiency could contribute to the aforementioned ALS-like syndrome associated with HIV infection (Sinha *et al.*, 2004; Rowland, 2011). Therefore, although the data are still sparse, a possibility remains that adaptive immune system immunodeficiency could contribute to ALS pathogenesis too.

Remaining challenges for translational ALS research

Particularities of immune responses in individual patients (due to their genetic makeup and/or environmental cues),

the dual roles of immune responses in ALS pathogenesis, and changes in these responses over the course of ALS, have rendered efficient targeting of pathogenic immune responses in ALS patients challenging. This can partially be attributed to the fact that historically most of the insight into the immune response in ALS came from the fast-progressing hSOD1 models, which do not necessarily replicate all the facets of the immune pathology observed in a wide spectrum of ALS patients (Wosiski-Kuhn *et al.*, 2019*a*). The same reasons contributed to the failure of many clinical trials; for example, celeoxib, a COX2 inhibitor, successfully slowed the disease progression in the SOD1 mouse model, but showed no efficacy in ALS patients (Pompl *et al.*, 2003; Cudkowicz *et al.*, 2006). On the other hand, treatment with tocilizumab, a blocking antibody against IL-6 receptor, decreased inflammation in sporadic ALS patients with high levels of inflammation, whereas it increased inflammation in patients with initially lower level of inflammation (Fiala *et al.*, 2013). This suggests that one of the major challenges facing efficient treatment will be accurate and time-dependent estimation of the level of immune system activation and inflammation.

In addition to evaluating peripheral inflammation, analysis of microgliosis and astrocytosis in patients can nowadays be done in real time with positron emission tomographic imaging using markers that target translocator protein, expressed primarily by activated microglia and astrocytes (Turner *et al.*, 2004; Corcia *et al.*, 2012; Alshikho *et al.*, 2018). Increased uptake of translocator protein markers is seen in precentral gyri of ALS patients, colocalizes with cortical thinning and correlates with more severe clinical phenotype and markers of neurodegeneration (Turner *et al.*, 2004; Alshikho *et al.*, 2016; 2018). However, this method is still of limited use for monitoring ALS progression because the binding capacity seems to plateau shortly after the appearance of symptoms. Therefore, more precise readouts are highly sought-after. Hence, to efficiently target immunity in ALS, we need better diagnostic approaches for estimating inflammation, procedures to optimize duration and dosing for a desired therapeutic effect, and larger clinical trials with better patient stratification and appropriate controls (Berry *et al.*, 2017; Wosiski-Kuhn *et al.*, 2019*a*; Goyal *et al.*, 2020).

Conclusion

ALS is an immune-mediated disease. Immune system dysregulation in ALS patients is present at multiple levels and many immune readouts including microglial activation, serum and CSF proteins, and blood cells are studied as potential therapeutic targets or biomarkers (Jeromin and Bowser, 2017; Beers and Appel, 2019). We have presented evidence that immune system dysregulation in ALS extends beyond excessive chronic inflammation, and that it may at some genetic backgrounds manifest as an inefficient immune response. We propose to classify the latter as an immunodeficiency, even though this term has been only rarely used in the context of ALS, and thus far only for T cell defects (Banerjee *et al.*, 2008; Seksenyan *et al.*, 2010). Immunodeficiency is an umbrella term for a variety of immune defects, ranging from adaptive to innate immunity, some of which lead to generally increased susceptibility to infection, whereas others are subtle and confer increased susceptibility to specific challenges. For example, haplodeficiency or dominant-negative effects of *TBK1* mutations are linked to susceptibility to herpes simplex encephalitis in children, without other obvious immune failures (Herman *et al.*, 2012). It may perhaps seem paradoxical that opposite immune dysfunctions such as excessive inflammation and immunodeficiency eventually lead to the same outcome. However, this is unsurprising because the inability to mount an appropriate immune response to eliminate cell debris, could lead to damage pile-up and thus trigger chronic inflammation. Indeed, an overlap between primary immunodeficiencies and inflammatory diseases has been reported for various inflammatory diseases such as inflammatory bowel disease, rheumatoid arthritis and others (Fodil *et al.*, 2016; Melamed, 2016). Perhaps the best example is chronic inflammation in Crohn's disease, which was initially regarded as an autoimmune disease, but is now thought to be an aftermath of defective production of immune factors, diminished immune cell recruitment and/or failed autophagy (Marks and Segal, 2008; Marks *et al.*, 2010). Similar ineffective microglial activation has been reported for Alzheimer's disease (Shi and Holtzman, 2018). Because of the wide plasticity of the immune system, manipulating immunity in ALS is a particularly attractive therapeutic approach. An experimental tyrosine-kinase inhibitor masitinib that targets signalling pathways in several innate immune cells showed promising results in a recent clinical trial (Mora *et al.*, 2019), and various immunomodulatory transplantation approaches are being tested (haematopoietic stem cells, Tregs, mesenchymal stem cells) (Mazzini *et al.*, 2003; Appel *et al.*, 2008; Mazzini *et al.*, 2018; Thonhoff *et al.*, 2018). However, these approaches are in the early stages of development and currently do not target specific subsets of patients. Rapidly progressing disease has been linked to an aggressive immune phenotype, suggesting that groups of ALS patients could be distinguished by their immunological readouts (e.g. Tregs, IL-6, microglial activation, etc.) (Brettschneider *et al.*, 2012; Beers *et al.*, 2017; Wosiski-Kuhn *et al.*, 2019*b*). Therefore, patient immune profiling will be crucial for selecting appropriate therapies. This will allow for overcoming the difficult task of dodging the Scylla and Charybdis of immune system activation in ALS—excessive inflammation and immunodeficiency—and possibly finding a sweet-spot for optimal immune responses for individual patients at specific disease stages.

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Competing interests

The authors report no competing interests.

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