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Deciphering the respective contribution of macrophages and microglia on motor neuron degeneration in Amyotrophic Lateral Sclerosis using Induced pluripotent stem cells

Elise Liu

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THESE

Pour obtenir de grade de docteur délivré par

SORBONNE UNIVERSITE

Ecole Doctorale Cerveau, Cognition et Comportement (ED3C)

Spécialité Neurosciences

Deciphering the respective contribution of macrophages and microglia on motor neuron degeneration in Amyotrophic Lateral Sclerosis using Induced pluripotent stem cells

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Le 07 Décembre 2021

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To Kening,

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ABBREVIATIONS

A

AD – Alzheimer Disease
AIS – Axonal Initial Segment
ALS – Amyotrophic Lateral Sclerosis
APC – Antigen presenting cells
Arg-1 – Arginine-1
ATF6 – Activation transcription factor 6
ATG7 – Autophagy related protein 7

B

BAI-1 – Adhesion G protein-coupled receptor B1
BDNF – Brain-derived neurotrophic factor
BiP – Binding immunoglobulin protein
bmdM – blood monocytes derived macrophages
BMAA – beta Methylanmino-L-Alanine
BMP4 – Bone morphogenetic protein 4

C

C1qa – Complement C1 subcomponent subunit A
C9orf72 – Chromosome 9 open reading frame 72
CCZ-1 – Vacuolar fusion protein
CD11b – Integrin Subunit Alpha M
CD11c – Integrin α X subunit
CD4+ Teff – CD4+ T effectors
CD58 – Lymphocyte function-associated antigen 3
CD86 – Cluster of differentiation 86
cGAS – Cyclic guanosine monophosphate-adenosine monophosphate synthase
CHMP2B – Charged multivesicular body protein 2B
CLRs – C-type lectin receptors
CNS – Central nervous system
CMA – Chaperone-mediated autophagy
CSF – Cerebrospinal fluid
CSF1 – Colony stimulating factor 1
CST – Cortico-spinal tract
CT – Computed tomography
CX3CL1 – fractalkine
CX3CR1 – C-X-3C chemokine receptor 1
CXCL11 – C-X-C motif chemokine 11
CXCL10 – C-X-C motif chemokine ligand 10

D

DAMPs – Damage associated molecular-patterns
DAP12 – DNAX-activating protein of 12-kDa
DENN – Differentially expressed in normal and neoplastic cells
DPR – Dipeptide repeats

E

eATP – Extracellular adenosine triphosphate
EB – Embryoid bodies
EEA1 – Early endosomal antigen 1
ELAM-1 – Endothelial leukocyte adhesion molecule 1
EMP – Erythromyeloid progenitors
ER – Endoplasmic reticulum
ERAD – Endoplasmic reticulum associated protein degradation
ERAP2 – Endoplasmic reticulum aminopeptidase 2
ERMCC – ER mitochondria calcium cycle
ERp72 – ER stress protein 72
ESC – Embryonic stem cells

F

FACS – Fluorescence activated cell sorting
fALS – Familial ALS
Fcrls – Fc receptor-like S, scavenger receptor
FGF – Fibroblast growth factor
FGF2 – Fibroblast growth factor 2
fHA – Fragment of hyaluronan
FL – Foetal liver
Flt3L – Flt-3 ligand
FTD – Frontotemporal Dementia

G

Gas6 – Growth arrest-specific protein 6
G-CSF – Granulocyte stimulating factor
GDP – Guanosine diphosphate
GEF – GDP/GTP exchange factors
GluRs – Glutamate receptors
GM-CSF – Granulocytes macrophages stimulating factor
GNLY – Granulysin
GOF – Gain of function
Gpr34, 56, 84 – Probable G protein coupled receptor 34, 56, 84
GRP78/BiP – ER chaperone glucose regulated protein 78
GTP – Guanosine triphosphate

H

Hexb – β -hexosaminidase subunit β

HLA – Human leukocyte antigen
HMGB1 – High-mobility group box 1
HRE – Hexanucleotide repeat expansion
HSC – Hematopoietic stem cells
HSPs – Heat shock proteins

I

IBA1 – Allograft Inflammatory Factor 1
ICAM-1 – Intracellular adhesion molecule 1
ICAM-3 – Intercellular adhesion molecule 3
IFN – Interferon
IFN- γ – Interferon-gamma
IFI16 – Interferon gamma-inducible protein 16
IGF1 – Insulin-like growth factor 1
IgG – Immunoglobulin G
IgM – Immunoglobulin M
IgSF – Immunoglobulin super family
IL-1,6,8,10, – Interleukin-1, 6, 8, 10
IL-34 – Interleukin 34
iMG – iPSC derived microglia-like cells
iMNs – iPSC derived motor neurons
iMPs – iPSC derived macrophages
iMye – iPSC derived myeloid progenitors like cells
iNOS – Inducible nitric oxide synthase
iPSc – Induced pluripotent stem cells
IRE1 – Inositol-requiring protein 1
IRF8 – Interferon Regulatory Factor 8
IRE1 α – Inositol-requiring protein 1 α
ITGB5 – Integrin Subunit Beta 5

J

Jun – Transcription factor AP-1

L

LAMP1, 2 – Lysosome-associated membrane protein-1, 2
LBHI – Lewy body-like hyaline inclusions
LC3 – Microtubule-associated protein 1A/1B-light chain 3
LOF – Loss of function
LPC – Lysophosphatidylcholine
LPS – Lipopolysaccharide
LTD – Long term depression
LTP – Long term potentiation

M

MACS – Magnetic activated cell sorting
MafB – Transcription factor MafB
MBL – Mannose-binding lectin
MCP-1 – Monocyte chemoattractant protein 1
mC9 – mutant C9orf72^{(G4C2)ⁿ}
M-CSF – Colony Stimulating Factor 1
MDA5 – Melanoma differentiation-associated gene 5
Mef2a – Myocyte-specific enhancer factor 2A
MERTK – MER Proto-Oncogene, Tyrosine Kinase
MFG-E8 – Milk fat globule EGF factor 8
mGluR – Metabotropic glutamate receptors
MHC-I – Major histocompatibility complex class I molecule
MHC-II – Major histocompatibility complex class II molecule
MICA – Major histocompatibility complex (MHC) class I chain-related protein A
MICB – MHC class I chain-related protein B
MIP-1 α – Macrophage inflammatory protein-1 alpha
MIP-1 β – Macrophage inflammatory protein-1 β
MMP – metalloproteinases
MN – Motor neuron
MSU – Monosodium urate
mTORC1 – Mammalian target of rapamycin complex 1
MYB – Myeloblastosis proto-oncogene

N

ND – Neurodegenerative Diseases
NES – Nuclear export signal
NF – Neurofilament
NF-H – Neurofilament heavy
NF- κ B – Nuclear factor-kappa B
NF-L – Neurofilament light
NF-M – Neurofilament medium
NGF – Nerve growth factor
NIID – Neuronal intranuclear inclusion disease
NKG2D – Natural killer group 2 member D
NLR – NOD-like receptors
NMHC-IIA – Non-muscle myosin heavy chain II-A;
NLS – Nuclear localisation signal
NMJ – Neuromuscular junctions
NT3 – Neurotrophin 3

O

Olfml3 – Olfactomedin-like protein 3
OPTN – Optineurin

P

P2Ry13 , 12– P2Y purinoceptor 13 , 12
PAMPs – Pathogen associated molecular-patterns
PBMC – Peripheral blood mononuclear cells
PD – Parkinson’s Disease
PDI/PDIA1 – Protein disulfide isomerase
PERK – PKR-like ER protein kinase
PET – Positron emission tomography
PNS – Peripheral nervous system
preMac – Pre-macrophages
PRR – Pattern recognition receptors
PS – Phosphatidylserine
PI3P – Phosphatidylinositol-3-phosphate
PU.1 / SPI1 – Spi-1 Proto-Oncogene
Py – Per Year

R

Rab 1, 5, 7 – Ras-related protein 1, 5, 7
Rabs – Rab-GTPases
RAN – Repeat-associated non-AUG
rER – Rough endoplasmic reticulum
RGG – Multiple three glycine-arginine
RHI – Round hyaline inclusions
RIG-I – Retinoic acid inducible gene-I
RLR – RIF-I-like receptors
RNP – Ribonucleoproteins
RNS – Reactive nitrogen species
ROS – Reactive oxygen species
RUNX1 – RUNX Family Transcription Factor 1

S

S1P – Sphingosine-1-phosphate
S100A12 – Protein S100-A12
sALS – Sporadic ALS
Sall1, 2 – Sal-like protein 1,2
SCF – Stem cell factor
SIGLEC-10 – Sialic acid binding Ig like lectin 10
Siglec-H – Sialic acid binding Ig-like lectin H
SIRP α – Signal regulatory protein α
Slc2a5 – Solute carrier family 2 facilitated glucose transporter member 5
SLI – Skein-like inclusion
SMRC8 – Smith-magenis syndrome chromosome region, candidate 8
Socs3 – Suppressor of cytokine signalling 3
SOD1 – Superoxide dismutase 1

SorCS2 – Sortilin-related receptor CNS expressed 2
SPP1 – Secreted phosphoprotein 1
SQSTM1 – Sequestosome 1
STING – Stimulator of interferon genes

T

TAM – Termed axon tract associated microglia
TARDBP – Transactive response DNA binding protein
TBK1 – Tank-Binding kinase 1
TGF β 1 – Transforming Growth Factor Beta 1
TGF β R1 – TGF-beta receptor type-1
TIM – T cell immunoglobulin mucin
TLR – Toll like receptor
Tmem119 – Transmembrane Protein 119
TNF – Tumor necrosis factor
TNFR1 – Tumor necrosis factor receptor 1
TNFR2 – Tumor necrosis factor receptor 2
TPO – Thrombopoietin
Tregs – Regulatory T lymphocytes
TREM2 – Triggering receptor expressed on myeloid cells 2
TSPO – Translocator protein
TYROBP – Transmembrane Immune Signaling Adaptor

U

UBQLN2 – Ubiquilin 2
ULBPs – UL16 binding proteins
UMNB – Upper motor neuron disease burden
UPR – Unfolded protein response
UPS – Ubiquitin proteasome system

V

VAPB – Vesicle-associated membrane protein-associated protein B
VCP – Valosine
VEGF – Vascular endothelial growth factor

W

WDR41 – WD repeat domain 41
WT – Wild-type

X

XBP1 – Spliced X-box binding protein 1

Y

Ym1 – Chitinase-like 3 protein

YS – Yolk-sac

INTRODUCTION

1- Amyotrophic Lateral Sclerosis disease

Amyotrophic Lateral Sclerosis (ALS), also known as Lou Gehrig's disease or Charcot disease, is a fatal progressive neurodegenerative disease affecting motor neurons (MNs) in the motor cortex, brain stem motor nuclei and in the anterior horn of the spinal cord leading to progressive paralysis. The term "A-myo-trophic" stands for "no-muscle-nourishment" and takes root from Greek. "Lateral" characterises the lateral column of the spinal cord in which MNs from the cortex lay their axons and where the degeneration occurs. "Sclerosis" was used to describe the hardening and scarring of the tissues in post-mortem examinations. Taken together the term which was given a century ago, still describes with accuracy the underlying pathology of ALS.

1.1 Clinical description of ALS

1.1.1 Jean-Martin Charcot's lesson

Jean-Martin Charcot is a French neurologist from the 19-20th century, clinical professor of nervous diseases at the faculty of medicine of Paris and academician. He was appointed head of service at the Salpêtrière in 1861, and devoted a large part of his time to the census and examination of his patients. From 1866, Charcot gave public courses at the Salpêtrière, which were extremely successful and attracted more and more students. Notably in 1869, he described Amyotrophic Lateral Sclerosis, a neurodegenerative disease to which his name will remain attached (Goetz 2000).

Jean-Martin Charcot is credited as the father of ALS not for having discovered the disease but for having gathered a few decades of clinical case studies and for making correlations between different observations made by others in a single syndrome. He has dedicated his career to understanding the underlying mechanism leading to this paralysis. His research has not only helped to identify ALS as a unique syndrome but also contributed to the understanding of spinal cord and brain stem anatomy and the organisation of the nervous system.

1.1.2 Understanding of the motor system

The motor system is a complex functional organisation, which implies the central nervous system (CNS) and the peripheral nervous system (PNS) (Figure 1, Figure 2). The human motor cortex is

located behind the frontal lobe just before the central sulcus. It is subdivided into two main areas, the primary motor area (Brodmann area 4) and the Brodmann area 6 which is located just in front of the area 4 and composed of the premotor cortex and medially the supplementary motor area (which will not be detailed herein) (Purves et al. 2001) (Figure 1) .

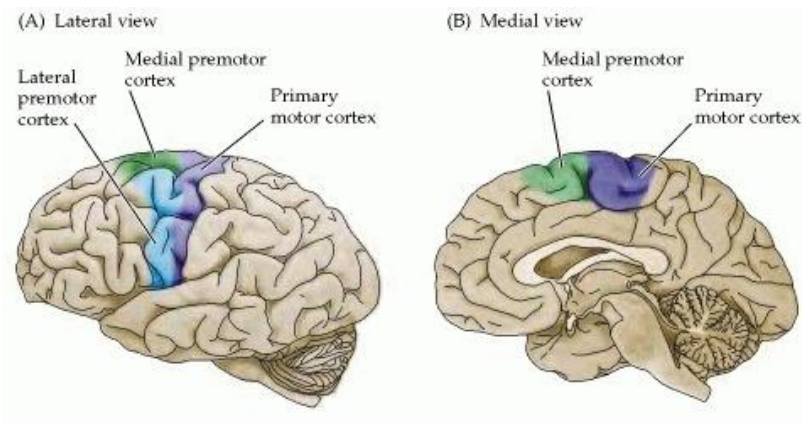


Figure 1 Illustration of motor area in the human brain. The primary motor cortex (Purple, Brodmann area 4) and premotor area (green and light blue Brodmann 6) are presented from two different views (A) Lateral (B) Medial. Image from Purves et al., 2001

The motor cortex is interconnected with the basal ganglia and the cerebellum, which convey regulatory inputs necessary for precision and coordinated movements Figure 2. Although the term motor cortex englobes all these cortical regions, it is most often used to identify primary motor cortex, which is the main relay to the lower MNs of the brainstem and spinal cord. The cortical MNs (or upper MNs) are the pyramidal cells of the cortical layer V also referred to as Betz cells. The latter's soma are located in the primary motor cortex and display long dendrites, which will branch into layer 1 (in orange on Figure 2). These cells are integrating the excitatory and inhibitory stimulus received from different brain regions and translate it into a signal which initiates or inhibits a voluntary movement (Zayia and Tadi 2021; Jara et al. 2014). Axons of these Betz cells reach the brainstem or spinal cord through corticobulbar or cortico-spinal tracts (CSTs), passing

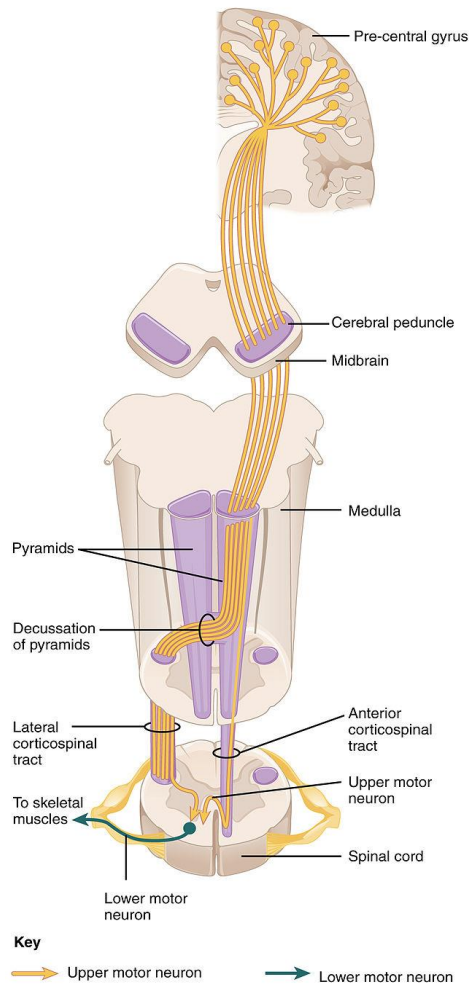


Figure 2. Illustration of the cortico-spinal tract (CST) conveying motor signals from motor cortex to skeletal muscles. The motor cortex and CSTs degenerate in ALS. From Wikipedia

through the internal capsule of the forebrain, the cerebral peduncle of the midbrain and running through the base of the pons (Figure 2). Upper MNs which innervate cranial nerve nuclei, the reticular formation and the red nucleus (constituting the indirect pathway which will not be presented herein), leave the brainstem at the appropriate level to synapse onto the lower MNs which innervate specific muscles. A large majority (90%) of the rest of the upper MNs decussate at the caudal end of the medulla to enter the lateral columns of the spinal cord, forming the lateral CST. The latter forms the direct pathway from the cortex to the spinal cord ending in the lateral portion of the ventral horn and intermediate grey matter where they directly synapse onto the spinal MNs (or lower MNs, in green on Figure 2) in the anterior horn of the spinal cord. The pyramidal tract axons, which have, not decussate at the medulla, continue ipsilaterally and terminate either on the same side or cross the midline via spinal cord commissure to reach the contralateral side of the spinal cord. These axons comprise partly the ventral CST, and are responsible for axial and proximal muscle movements and controls.

The lower MN is responsible for transmitting the signal from the upper MN to the effector muscle to elicit movement. According to their targets, lower MNs are divided into 3 categories: somatic, branchial and visceral.

- Somatic MNs are innervating skeletal muscles, involved in voluntary movements. They are subdivided into three types: alpha, beta, and gamma efferent MNs.
 - Alpha MNs innervate extrafusal muscles fibres (which are the main force-generating fibres of the muscles). A single MN can synapse onto 150 muscle fibres on average (Heckman and Enoka 2004) constituting a motor unit. The

soma of alpha MNs is located in the anterior horn of the spinal cord and directly receives signals from the upper MNs. Besides controlling muscle contraction, alpha MNs along gamma MNs are also responsible for muscle tone which is the continuous force generated by non- contracting muscle to oppose stretching (Zayia and Tadi 2021)

- Beta MNs innervate intrafusal fibres of muscles spindles and some collaterals to extrafusal fibres.
- Gamma MNs are innervating intrafusal fibres found within the muscle spindles. Despite their name MNs, they are not directly causing any motor function.
- Branchial MNs are involved in facial expression, mastication, swallowing and phonation (Zayia and Tadi 2021).
- Visceral MNs are disynaptic conversely to somatic and branchial MNs. They first synapse onto neurons of the ganglia of the autonomic nervous system, which themselves will directly synapse onto appropriate muscles. Visceral MNs are innervating cardiac muscle and smooth muscles of the viscera (Zayia and Tadi 2021).

1.1.3 Bedside diagnosis examination

Since several years, ALS and frontotemporal dementia (FTD) disorders are considered two ends of a spectrum of neurodegenerative diseases (ND). FTD is a type of dementia characterised by impaired judgment and executive skills (Neumann et al. 2006; Burrell et al. 2016; Liu et al., 2021 review submitted). ALS shares clinical and pathological features with FTD.

Clinically ALS is characterised by a progressive degeneration of upper and lower MNs leading to paralysis of patients. Patients usually first present complaining about progressive weakness. The latter manifests often unilaterally in a single segment (i.e. bulbar, cervical, thoracic or lumbosacral) and is in a majority of cases reported in the extremity of one limb. Importantly, ALS patients do not present with sensory defects, pain, bowel or bladder problems (Ferguson and Elman 2007). Overtime, the reported weakness may extend in the segment, and spread over to the contralateral side slowly affecting other body's regions. Bulbar dysfunction (i.e. dysarthria, dysphagia) is reported in one third of ALS patients, and respiratory onset is the less common pattern of presentation (Ferguson and Elman 2007). Across disease progression, patients may encounter

fasciculations, muscle cramps, mood swings, and at a later stage of the pathology, difficulties in breathing are recurrent usually necessitating breathing assistance. Cross sectional studies performed over the last decade identified cognitive impairments associated with FTD present in 50 % of ALS patients (Burrell et al. 2011; Giordana et al. 2011; Lomen-Hoerth et al. 2003). Conversely, up to 30% of FTD patients develop motor dysfunction (Burrell et al. 2011; Lomen-Hoerth et al. 2003).

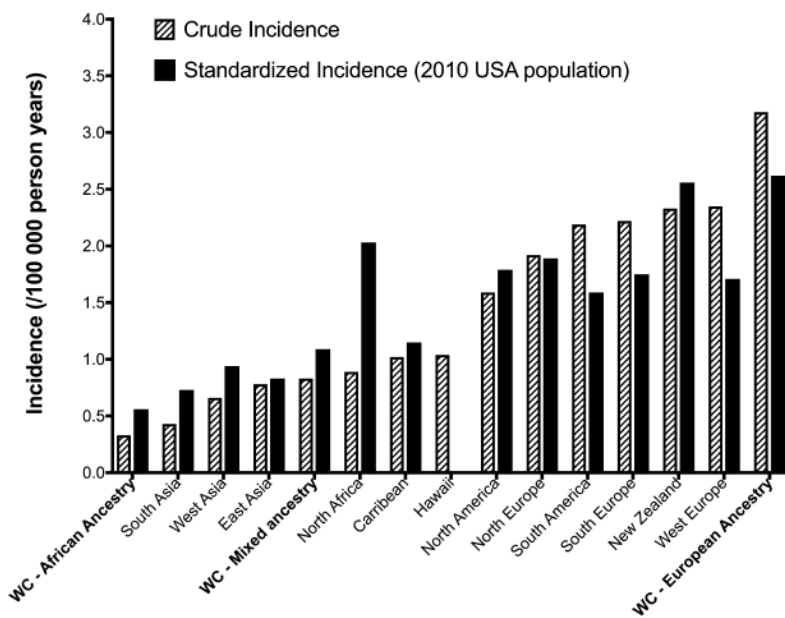
The diagnosis of ALS is challenging, as clinical presentations and disease evolution are very heterogeneous between patients. The diagnosis is mostly based on clinical presentation; no single test confirms ALS diagnosis. Nonetheless, neuroimaging and electrophysiological studies serve as the basis for implementing evidence for ALS diagnosis. As ALS is a progressive pathology, diagnosis is often based on several consultations separated in time, with the mean time from onset to diagnosis being 10.1 months (Nzwalo et al. 2014). Although more and more biomarkers are proposed as early characteristics of ALS pathology and could serve for diagnosis, yet they are still not routinely used (Verde et al. 2019; Lu et al. 2015; Shinotoh and Armon 2020). The current standard for clinical diagnosis is Revised El Escorial World Federation of Neurology Criteria, which permit classification of patients into different classes such as suspected, possible, probable or definite ALS (Brooks 1994). For definite ALS diagnosis, upper and lower signs are necessary. Lower motor damage includes flaccid paralysis, cramps, fasciculation, muscle atrophy, weakness and hyporeflexia. In the bulbar region, the main signs include dysarthria, poor palatal elevation, weak tongue pulsion, atrophy and fasciculations accompanied by facial weakness (Ferguson and Elman 2007). Upper motor degeneration is more difficult to detect, it includes slowness of movements, no muscle atrophy, increased muscle tones and hyperreflexia (Ferguson and Elman 2007). Babinski signs, named after the neurologist Joseph Babinski, is a routinely used clinical test to assess possible upper MNs lesions constituting damage to the CST. The Babinski response is characterised by an abnormal reflex (big toe extension) when the sole of the foot is stimulated with a blunt instrument (Ambesh et al. 2017). In 30-50% of ALS cases, Babinski signs are observed attesting an upper MN lesion. In the bulbar region, upper motor signs include slow and spastic speech, declined tongue movements, palmomental sign, jaw jerk reflexes and exaggerated or asymmetric facial reflexes (Ferguson and Elman 2007). Genetic testing may also be proposed if familial history

is present for the patient, and can help to exclude some ALS mimicking diseases (e.g. late onset Spinal Muscular Atrophy, Kennedy's disease).

1.1.4 Epidemiology of ALS

ALS worldwide incidence

Worldwide ALS incidence, corresponding to the number of new cases per year, is between 1.59-1.69 per 100 000 person-year (py) (L. Xu et al. 2020; Marin et al. 2017), however, a heterogeneity of the incidence can be observed depending on the continent and ethnicity. In fact, a meta-analysis including 44 studies, covering 45 geographical areas and 11 subcontinents revealed the high heterogeneity amongst regions. The Table summarises the crude incidence obtained from this meta-analysis. Certain incidence have to be taken with a grain of salt as some regions are poorly documented (Oceania n=1, South Asia (Iran) n=1, West Asia (Israel) n=1, North Africa (Libya) n=1, South America n=2, East Asia n=3). The highest incidence is seen in Western countries (Europe: 1.92-2.35/ 100 000py; North America: 1.59/100 000 py; Oceania: 2.33/100 000 py), with the exception of South America where a very high incidence is also reported (2.19/100 000py). Asia have a low level of ALS crude incidence (0.43-0.78/100 000py), however further studies would be needed to confirm this low incidence (Marin et al. 2017). Little is known regarding the epidemiology of ALS in Africa. The latest prospective incidence study in South Africa report a crude incidence rate of 1.09/100 000 py (Henning et al. 2021). While the North Africa based on one



study, report a crude incidence of 0.89 in Libya (Marin et al. 2017). Thus, the incidence in this region appear lower than Western countries but higher than Asian countries (Figure 3 , Table).

Figure 3 Worldwide ALS crude and standardized/adjusted incidence rates (IRs). The 2010 USA population was used as standard population. The figure shows from

the highest to lowest incidence: higher incidence in Western countries, followed by African and lastly Asian countries. Interestingly, the authors showed that European ancestry confer a higher risk in developing ALS compared to mixed or African ancestry demonstrating also a genetic background heterogeneity between the populations. The figure is extracted from Henning et al., paper from 2020 the authors adapted the figure from a previous article from Marin et al., 2017.

Some ALS clinical features are also heterogeneous such as survival time from onset. In Asia differences were observed amongst regions (in some regions only very few data are available) with longer survival in Iran (48 months) in comparison to Israel (36 months) or East Asia (28 months). In Europe, survival ranges from 23-49 months, with shorter survival in Northern Europe (around 25 months) compared to Western and Southern Europe (~ 30 months) (Marin et al. 2016).

Table 1 Summary of ALS epidemiology in different continents. The results are based on Marin et al., 2017, Marin et al., 2016, Marin et al., 2018, Henning et al., 2020

| | ALS incidence / 100 000 py | Survival time from onset (months) | Mean Age at ALS onset and diagnosis (years) | Age specific incidence Peak age (years) – Incidence Rate (py) |
|---------------|---------------------------------------|--------------------------------------|---|---|
| Europe | 1.92 North 2.22 South 2.35 West | ~25 North ~ 30 South ~ 30 West | ~ 63 North ~ 65 South ~ 65 West | 71.6 – 6.98 North 74.6 – 7.46 South 74.9 – 8.17 West |
| North America | 1.59 | ~ 32 | ~59 | 77.4 – 7.77 |
| South America | 2.19 | ? | ~57 | ? |
| Asia | 0.78 East 0.43 South 0.66 West | ~ 28 East ~ 48 South ~ 36 West | ~ 59 East ~ 54 South ~56 West | 74.3 – 2.20 East |
| Africa | 0.89 North 1.09 South | ? | ~47 North | ? |
| Oceania | 2.33 | ? | ~ 65 | ? |

ALS disease age of onset

The mean age at ALS onset is also subject to variabilities between continents contiguously to life expectancy in men and women. In Europe, the median value of the mean age at onset is between

63-65 years of age, while for North America and East Asia medians were around 59 years of age (Table) (Marin et al. 2016). Age specific ALS pattern was also shown to be different between the continents. Interestingly, the question regarding if ALS is an age related or an ageing related disorder is still a debated question. A meta-analysis associating worldwide ALS incidence rates and age showed that the pattern of age specific incidence was similar between Europe and North America with a peak of incidence in Europe between 6.98- 8.17/ 100 000 py between 71.6-74.9 years of age, in North America the peak of incidence was at 7.7 /100 000 py at 77.4 years of age. In East Asia the peak of incidence is about 2.2/100 000 py at around 74.3 years of age (Marin et al. 2018). This shows a reduction of a factor 3 between Caucasians and Chinese in the risk of developing ALS in the range of age with higher incidence. Altogether, the risk of developing ALS peaks between 50-75 years of age and decreases thereafter. This suggests that ALS may rather be an age dependent disease although further studies would be needed (Hardiman, van den Berg, and Kiernan 2011).

Gender effect

Risk of developing ALS seems also to have a gender effect. In Europe ALS is predominant in men compared to women by a ratio of 1.25:1, while in North America and New Zealand the pooled estimates are respectively 1.33, 1.22 and reaches the higher ratio in Asia (East 1.53, West 1.72). (Logroscino et al. 2010; Marin et al. 2016).

Clinical features heterogeneity

Regarding clinical features differences, bulbar onset is more common in Northern Europe (45.4%) compared to other European subcontinents (34.9%) and to Western and Southern Europe (34.2%), with the lower proportion being in North America, East Asia, Israel and Iran (15%) (Marin et al. 2016).

Taken together, ALS epidemiology is very heterogeneous depending on the continent and the ethnicity (Marin et al. 2017). Some regions are very poorly documented and thus some information are lacking in order to draw clear conclusions. The heterogeneity observed need also to be linked to differences in health system diagnosis, life expectancy, environmental factors, site of onset and probably the underlying genetics.

1.2 The importance of genetics in ALS

1.2.1 Heterogeneity of ALS genetics

While the majority (80-95%) of ALS cases are sporadic (sALS), meaning without any familial history of the disease, around 5-20% of ALS patients have a familial history of the disease (fALS), meaning a record of the disease in the family (Figure 5).

Most genetic causes have a dominant mode of inheritance and incomplete penetrance. Since 1993, more than 40 genes have been identified as causative of or highly associated with ALS (Gregory et al. 2020). Thanks to advances in sequencing technologies, several rare variants linked to ALS disease and genetic risk factors have been uncovered in the past few years (Gregory et al. 2020) (Figure 4). These discoveries undeniably complexify the genetic landscape of ALS disease.

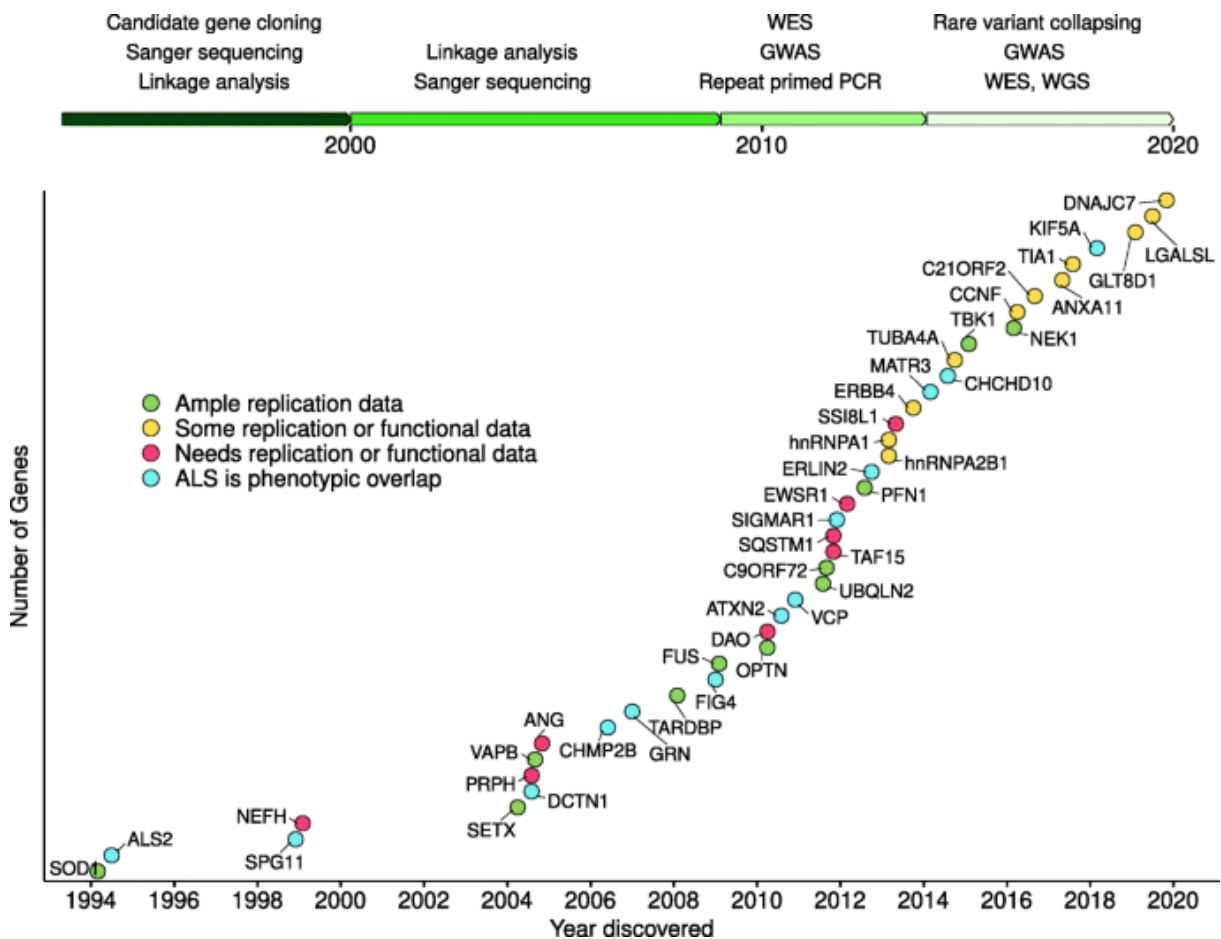


Figure 4 Identified genes which have been linked to ALS across the years. The different ALS genes identification strategies are presented above. Strategies have evolved from individual gene mapping to increasingly powered technological and statistical methodologies. Since the implication of SOD1 in 1993, 42 additional genes have been

implicated in ALS to date. Some genes still need to be replicated with other genetic support and in functional studies in order to validate their linkage with ALS pathology. GWAS, genome-wide association studies; WES, whole-exome sequencing; WGS, whole-genome sequencing. Image is extracted from Gregory et al., 2020

Mutations in these ~40 genes account for 25-35% of familial cases, and 5-10% of sporadic cases (Renton, Chiò, and Traynor 2014). With the advancement of sequencing techniques and the increase in the number of cohorts, a more complete characterisation of gene mutation frequencies in familial and sporadic patients has revealed significant differences between ancestries (Zou et al. 2017). Typically, around 60% of European families with ALS are explained by known genes (C9ORF72 >> SOD1 >> TARDBP> FUS), while in Asia the 40% of families currently explained have a different genetic distribution (SOD1 >>>FUS> TARDBP> C9ORF72). Furthermore, mutations in these same genes can be found in around 8% of sporadic European patients (C9ORF72 >>>> SOD1 >> TARDBP> FUS) and only 3% in Asian populations (SOD1 >> FUS> TARDBP = C9ORF72) (Zou et al. 2017, Figure 5).

Additionally, the explosion in sequencing speed and the diminishing cost led to sequencing of a larger number of genes in increasing numbers of ALS patients. These methods also showed that part of the ALS population carried potentially pathogenic variants in more than one ALS related gene and possibly represented oligogenic inheritance (Cady et al. 2015; van Blitterswijk et al. 2012; Kuuluvainen et al. 2019). The latter, refers to the concept that certain diseases are not inherited as monogenic Mendelian disorders, but they rather correspond to a pattern in which mutations in a small number of genes can interact genetically to manifest a phenotype (Alsemari et al. 2018).

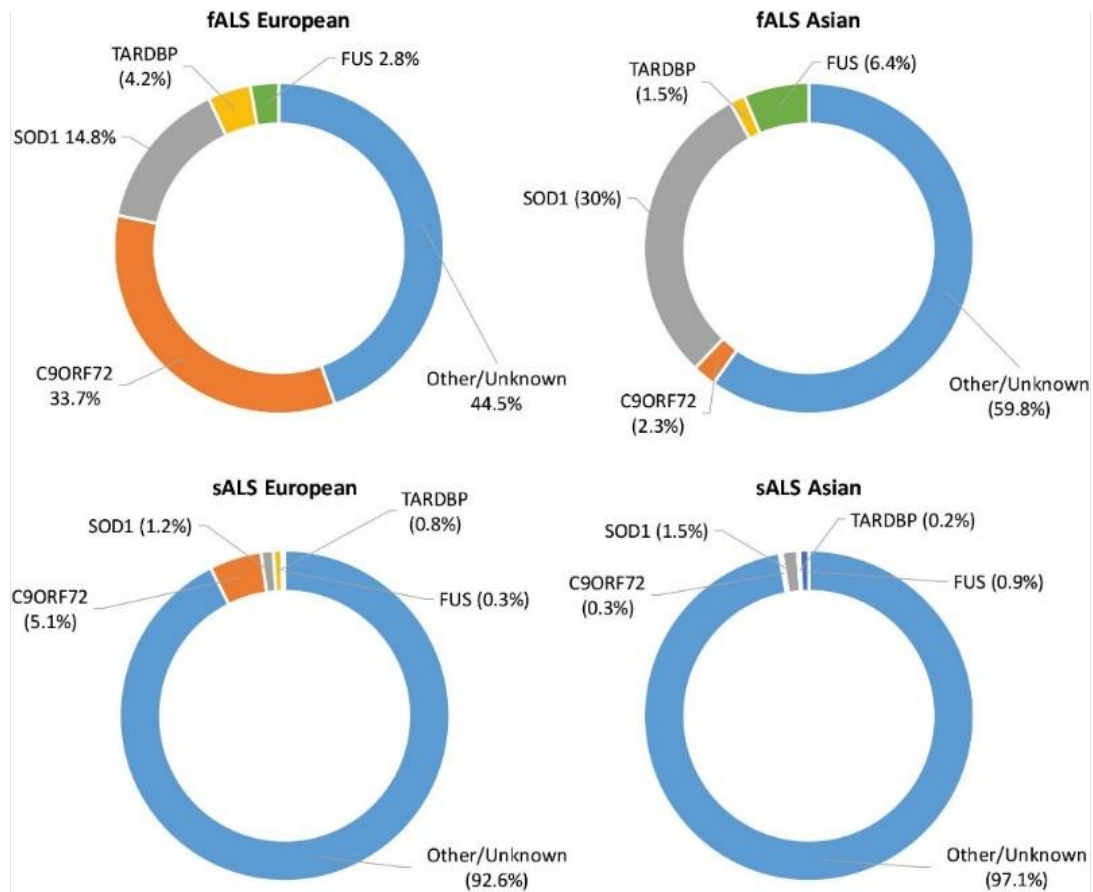


Figure 5 Proportion of the ALS cases explained with the fourth most commonly mutated genes in Asian and European populations. This figure shows the heterogeneity of familial and sporadic ALS genetic between European and Asian populations. The image is from Mejzini et al., 2019 data are adapted from the paper of Zou et al., 2017

Interestingly, when looking at the function uncovered by the different ALS linked genes, it is observed that most of them are part of important canonical pathways and are genes ubiquitously expressed. A simplified classification has been proposed for these genes based on their function, and they were observed to fall into three major families of genes implicated in: protein homeostasis, RNA-binding proteins and cytoskeletal proteins (R. H. Brown and Al-Chalabi 2017).

Taken together, advances in high throughput DNA sequencing technologies have contributed to an acceleration in identification of new disease associated variants and genetic risk factors for fALS and sALS. While comprehensive ALS genes findings continues to uncover individuals with possible oligogenic inheritance of ALS. Many newly identified genetic risk factors are common between several other ND suggesting a possible overlap in pathological processes between different ages related diseases.

1.2.2 Main ALS Genetic causes

Superoxide dismutase 1 (SOD1)

Copper-Zinc superoxide dismutase 1 (SOD1) was the first gene identified in 1993 to be linked to ALS (Rosen et al. 1993). The latter encodes a 32kDa homodimeric metalloenzyme, which helps to catalyse the dismutation of the toxic superoxide anion O_2^- to O_2 and H_2O_2 . The protein is expressed in the cytosol, nucleus, peroxisomes and more debated, in mitochondria (Tafari et al. 2015). SOD1 protein is highly conserved throughout evolution, with more than 50% homology between human and non-human mammalian species (Bordo, Djinovic, and Bolognesi 1994).

SOD1 mutations account for about 20% of fALS and 2-3% of sALS cases (www.alsod.org), with generally an autosomal dominant mode of inheritance (Exception is from the recessive D90A variant of Swedish and Finish origins (Andersen et al. 1995)). To date more than 200 variants are identified (Andersen et al. 2003; Valdmanis et al. 2009; Zinman et al. 2009; Bunton-Stasyshyn et al. 2015; Muratet et al. 2021, Figure 6), with most of them being missense mutations, and the remaining being non-sense and deletions conducting to changes in the length of the SOD1 protein. SOD1 variants are differentially distributed between ethnicity and worldwide populations. As a matter of fact, they account for about 30% of fALS in Asia, 23,5% in the US, 23,5% in Scandinavian, 21% in the UK, 14,3% in France and 19,4% in Italy (Battistini et al. 2005; Gellera et al. 2001; Andersen et al. 2003; Muratet et al. 2021).

In most cases, SOD1 patients develop the pathology earlier than other ALS forms, nonetheless phenotype, disease duration and severity of the disease differ significantly between variants (Millecamps et al. 2010; 2012). Rapid disease course (<3 years) and shorter survival times are associated with A4V, H44R, H43R, L84V, G85R, N86S and G93A mutations (Saeed et al. 2009; Bali et al. 2017; Bernard et al. 2020); whereas D83G, L144F, G93C, D90A, H46R have slower disease progression and better long-term survival (up to 44 years for L144F (Weber et al. 2012)). At the clinical level, genotype-phenotype correlations are also dependent on the particular variants. However, bulbar onset is less frequent in SOD1 patients compared to other fALS and they rarely present with cognitive impairments (H.-F. Li and Wu 2016).

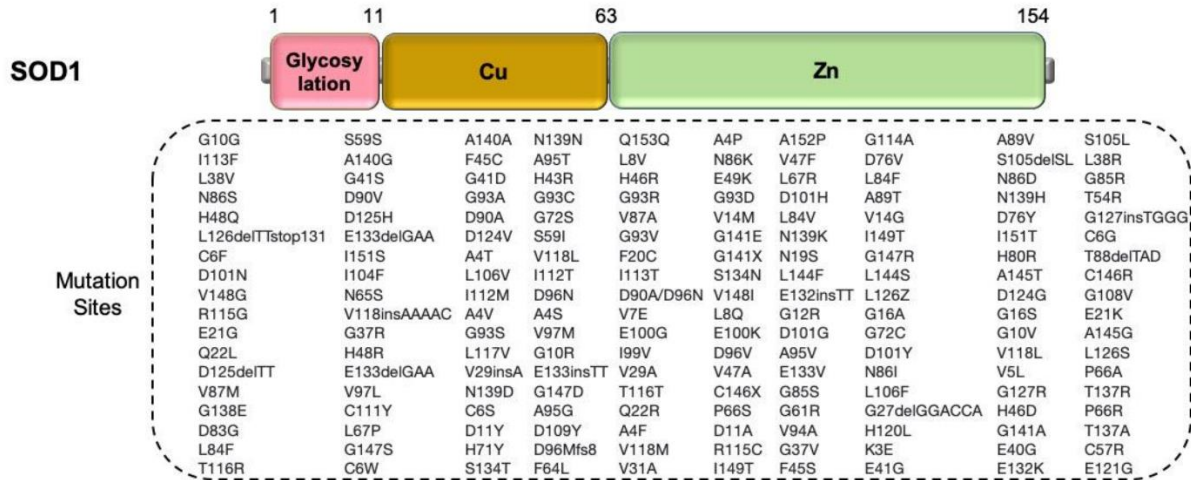


Figure 6 Schematic overview of SOD1 protein structure (at the top) and the different variant sites in SOD1. The data were extracted from the ALS online database (ALSoD) from Yousefian-Jazi et al., 2020 and initially from Abel et al., 2012. The figure is from the paper of Yousefian-Jazi et al., 2020

Concerning SOD1 pathomechanisms, all lines of evidences tend to suggest that SOD1 mutations lead to a toxic gain of function rather than a loss of function. As a matter of fact, over-expression of human G93A SOD1 transgene in wild type mice led to ALS-like pathology despite the presence of the endogenous murine SOD1 enzyme (Gurney et al. 1994). While knockout of SOD1 gene in mice did not recapitulate ALS pathology (Shefner et al. 1999). Interestingly, elevation of wild-type SOD1 (wt-SOD1) protein expression in mice do not lead to development of apparent motor phenotypes (Bruijn et al. 1998). Under physiological conditions, the SOD1 functions as a homodimeric enzyme with two subunits composed of beta-barrel core and seven loops at the edge, held together via intramolecular di-sulfide bonds. Each subunit is composed of a binuclear Cu/Zn site which is important for enzymatic activity (Valentine, Doucette, and Zittin Potter 2005). SOD1 maturation implies binding of copper and zinc ions and di-sulfide bonds formation, this is a complex process taking place in the cytosol (Huai and Zhang 2019). Although the properties of SOD1 variants are very different in terms of protein stability, catalytic activities and metal binding, it seems that most of the SOD1 variants are subject to retard in post-translational maturation, which generates unstable SOD1 protein prone to misfolding (Tokuda and Furukawa 2016). Mutation-induced conformational and functional changes of SOD1 have been suggested to confer the gain of toxicity via interaction with other proteins and in several mechanisms. A debate remains regarding whether the soluble or aggregated forms of the protein are responsible for exerting toxicity or if it is a protective mechanism. An extensive review summarises the different

interacting partners of mutant SOD1 in the cells and the impacts these bindings may have on other functions than the basic detoxifying function of SOD1 (Huai and Zhang 2019). These mechanisms include excitotoxicity, oxidative stress, endoplasmic reticulum stress (ER-stress), mitochondrial dysfunction and prion like propagation (reviewed by Hayashi, Homma, and Ichijo 2016).

Chromosome 9 open reading frame 72 (C9orf72)

In 2011, two separate groups identified a large hexanucleotidic (GGGGCC) repeat expansion (HRE) in the first intron of *C9orf72* gene, located on the short arm of chromosome 9. These expansions were found to segregate with high numbers of both familial, sporadic ALS cases as well as FTD patients (Renton et al. 2011; DeJesus-Hernandez et al. 2011). The discovery of mutation in this gene was the first strong evidence supporting an association between ALS and FTD pathologies. At the time the gene was discovered, the exact function of C9orf72 gene was unknown. Since then computational analyses showed structural homology between C9orf72 and Differentially Expressed in Normal and Neoplastic cells (DENN) proteins, the latter being Guanosine Di and Triphosphate (GDP/GTP) exchange factors (GEFs) that activate Rab-GTPases (Rabs) (Levine et al. 2013; D. Zhang et al. 2012). These factors are known to be involved in membrane trafficking events via activation of Rabs. As C9orf72 colocalised with several of these Rabs it was suggested that it might be involved in endocytic and phagocytic trafficking (Farg et al. 2014; Y. Aoki et al. 2017). More recently, C9orf72 have been shown to also regulate lysosomal trafficking and notably lysosomal exocytosis in macrophages and microglia (Y. Zhang et al. 2018; Amick, Rocznik-Ferguson, and Ferguson 2016; Amick and Ferguson 2017; Corriero and Horvitz 2018). Moreover, C9orf72 has also been shown to be implicated in immune systems (Atanasio et al. 2016; Burberry et al. 2016; Sudria-Lopez et al. 2016; McAlpine et al. 2018) as well as such as stress granule formation, nucleocytoplasmic transport and RNA binding protein metabolism (Maharjan et al. 2017; K. Zhang et al. 2015; Haeusler et al. 2014).

Typically, 2-23 copies of the HRE are present in the gene of normal subjects, but ALS patients present with length of the expansions varying between 700–1600 repeats (Cooper-Knock et al. 2012; DeJesus-Hernandez et al. 2011). Longer length of expansions seemed to inversely correlated with gene expression level (Van Mossevelde et al. 2017). In ALS, these expansions represent the first genetic cause, explaining around 40% of fALS, and around 8% of sALS (Pliner, Mann, and Traynor 2014), and is transmitted in an autosomal dominant mode of inheritance with a suggested

age dependent penetrance (Majounie et al. 2012). The mutation frequency is differentially expressed between ethnic groups and geographic location. Caucasian in Europe and North America have the highest frequency of C9orf72 HRE in the ALS population, ranging from 2.4 % in France to 50% in Denmark. In Asian ALS populations, the frequency of c9orf72 HRE is rare with the higher percentage in Japon (1.2%) and the lowest in China, Korea and India (nearly 0%) (Ogaki et al. 2012; Majounie et al. 2012).

At the clinical levels, C9orf72 mutated patients are associated with a wide range of diversity. Patients can either develop FTD, ALS or both. The age at onset pathology is highly heterogeneous ranging from 28-83 years old, with a mean age being similar to that of SOD1 mutated patients (Majounie et al. 2012; Adriano Chiò et al. 2012; Sabatelli et al. 2012; Byrne et al. 2012; Millecamps et al. 2012). Patients present dominant bulbar involvement compared to other ALS forms, and often present cognitive and behavioural impairments (B. N. Smith et al. 2013). The disease progression rate is fast ranging from 3-96 months in most cases, and is around 20 months from symptoms appearance (Millecamps et al. 2012; Cooper-Knock et al. 2012).

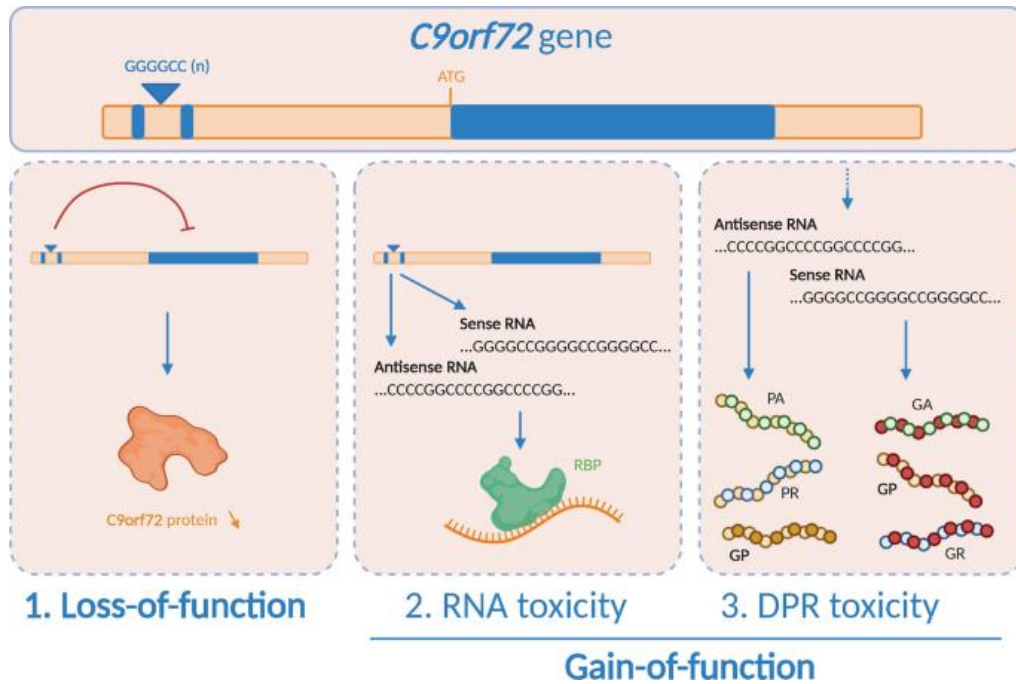


Figure 7 The three disease mechanisms proposed to underlie C9orf72 ALS. Firstly, the HRE could impede transcription processes leading to a reduction in the protein levels and thus a loss of function of the gene. Secondly, bidirectional transcription of the HRE may generate secondary RNA structures termed RNA foci, which could sequester regulatory RNA binding proteins (RBPs). Lastly, the non-ATG repeat associated (RAN) translation process of these sense and antisense RNA structures produce potentially toxic dipeptide repeats proteins (DPR) from sense (Glycine-proline (GP),

Glycine-Alanine (GA), Glycine-Arginine (GR)) and antisense (GP, proline-Alanine (PA), Proline-Arginine (PR)) strands. Figure from Braems et al., 2020

The suggested most widely studied non-exclusive pathomechanisms of C9orf72 are of three types (Figure 7). The first, consist of a loss of function (LOF) due to HRE causing epigenetic transcriptional silencing of the *C9orf72* gene. In fact, while mRNA level seem to be reduced by ~50%, protein levels are probably reduced only by 25% and there is no indication that the C9orf72 function is impaired in C9orf72 mutated ALS patients (Cooper-Knock et al. 2014; Waite et al. 2014; Braems, Swinnen, and Van Den Bosch 2020). Most studies using in vitro models to study the LOF mechanisms from C9orf72 HRE, found that the most important observations were the presence of autophagic alterations. Yet the latter cannot be demonstrated to be disease relevant and could be as well the cause of gain of function (GOF) mechanisms. In animal models, C9orf72 conditional knockout only present immune dysfunctions but no MNs degeneration thus suggesting that LOF is not a prevailing mechanism (Atanasio et al. 2016; Burberry et al. 2016; ORourke et al. 2016; Sudria-Lopez et al. 2016; McAlpine et al. 2018; Braems, Swinnen, and Van Den Bosch 2020). LOF alone is suggested to be insufficient to cause ALS pathology.

Secondly, two different GOF mechanisms are suggested. They are linked to RNA toxicity or dipeptide repeats (DPR) toxicity and are the most widely supported hypotheses to explain the pathology of C9orf72 in ALS. The underlying mechanism are linked to the bidirectionally transcription of HRE leading to sense and antisense transcripts termed RNA foci, additionally these transcripts can be translated to unexpected proteins termed DPR by repeat-associated non-ATG (RAN) translation mechanism. To determine the GOF mechanisms, several studies have overexpressed constructs containing either GGGGCC or CCCCGG repeats of various length and expression levels in model systems. Expression of these repeats in cultured neuronal cells, drosophila and mice produce RNA foci and DPR proteins, which cause some toxicity (Stopford et al. 2017; Mizielinska et al. 2014; J. Chew et al. 2015; 2019). Additionally, transgenic mouse models containing disease associated expansions (~100-1000 HRE) of human c9orf72 gene developed pathological features observed in C9 ALS patients, including RNA foci and Repeat-associated non-AUG (RAN) translation DPR (O'Rourke et al. 2015; Peters, Ghasemi, and Brown 2015; Y. Liu et al. 2016).

- The toxicity mediated by RNA Foci are suggested to be multiple ranging from sequestration of RNA binding factors, to nucleocytoplasmic transport deficits, perturbation of transport granule function or neuron branching defects (K. Zhang et al. 2015; Sareen et al. 2013; Y.-B. Lee et al. 2013; Cooper-Knock et al. 2014; Burguete et al. 2015; Aladesuyi Arogundade et al. 2019).
- Regarding the toxicity mediated by DPR proteins, it is less clear. DPR positive inclusions are observed in ALS patients as well as in transgenic mouse models of C9orf72 mediated disease (O'Rourke et al. 2015; Peters, Ghasemi, and Brown 2015; Y. Liu et al. 2016). The Glycine-Alanine (GA) DPR is the most visible in p62/ubiquitin positive inclusions in the brain and spinal cord of ALS and FTD patients, and c9-ALS mice (Y.-J. Zhang et al. 2016; May et al. 2014a). Expression of GA DPR in neuronal cultures conducts to reduced dendritic branching, ER-stress and proteasomal inhibition (Y.-J. Zhang et al. 2016; May et al. 2014a). GR/Proline-Arginine (PR) DPR were shown also to be toxic when expressed by plasmid transfection in several cell lines (Wen et al. 2014; K.-H. Lee et al. 2016; Tao et al. 2015). They are thought to be associated with nucleolar stress, impaired stress granule formation and impaired assembly, dynamic and function of membrane less organelles (Wen et al. 2014; K.-H. Lee et al. 2016; Tao et al. 2015). In the human pathology, the toxicity of these DPR remains to be determined.

Taken together, LOF mechanisms was shown to only poorly recapitulate ALS pathology, while different GOF mechanisms have grown in evidences of their implication of ALS pathology. With more insight and a better understanding of the toxicity of these different LOF and GOF mechanisms, new hypotheses are emerging suggesting synergistic mechanism in which LOF may exacerbate the GOF mechanisms and thus lead to neuronal degeneration. In fact, several recent studies support this multiple hit mechanism and would need further examination (Sellier et al. 2016a; Sullivan et al. 2016; Shao et al. 2019; Q. Zhu et al. 2020; Boivin et al. 2020).

Transactive response DNA binding protein (TARDBP)

In 2006, the *Transactive response DNA binding protein (TARDBP)* gene encoding TDP-43 protein was demonstrated to be the major constituent of ubiquitin positive inclusions in post mortem tissues, which is one of the hallmarks of ALS and FTD with ubiquitin positive inclusions (M. Neumann et al. 2006). TDP-43 accumulations in neuronal cells constitute a group of disease

termed TDP-43 proteinopathies, of which ALS is part. The hallmark of this group of diseases is the sequestration of TDP-43 in the cytoplasm constituting aggregates interacting with cellular machinery.

In 2008 TDP-43 mutations were shown to segregate with some fALS and sALS cases (Gitcho et al. 2008; Sreedharan et al. 2008; Kabashi et al. 2008). TDP-43 is an ubiquitously expressed 43kDa predominantly nuclear protein and is part of the family of heterogeneous Ribonucleoproteins (RNP), which interfere in multiple cellular functions such as transcription, RNA splicing and transport via its binding to DNA and RNA. Thus, TDP-43 has myriad key cellular interactors making it essential for proper cellular homeostasis (Reviewed by Prasad et al. 2019). TDP-43 is a highly conserved protein, composed of a N-terminal domain containing a Nuclear localisation signal (NLS), two RNA recognition domains, a Nuclear export signal (NES) and a C-terminal domain encompassing a prion-like domain which serve for protein-protein interactions (Figure 8) (Buratti and Baralle 2001; Cohen, Lee, and Trojanowski 2011; Lukavsky et al. 2013; Kitamura et al. 2018).

TARDBP mutations are present in 5-10% of fALS and <1% of sALS (Sreedharan et al. 2008; Van Deerlin et al. 2008; Rutherford et al. 2008; Corrado et al. 2009). To date more than 40 mutations have been identified in both fALS and sALS, with most of them found in the exon 6, which encodes the C-terminal glycine rich part of the protein (Figure 8). The majority of them are missense mutations, with a very few deletions giving rise to truncated proteins at the C-terminal. Population based studies revealed a higher proportion of *TARDBP* mutated cases in China (15,2% , sALS 0,73%) (Zou et al. 2012), and North England (5% fALS, 0,4% sALS) (Kirby et al. 2010) populations compared to North European population (2,7% vs 1% , Corrado et al. 2009). Clinically TDP-43 mutated patients often present typical bulbar or limb onset, very variable age at disease onset as well as variable disease progression and rare cognitive involvements (Kirby et al. 2010).

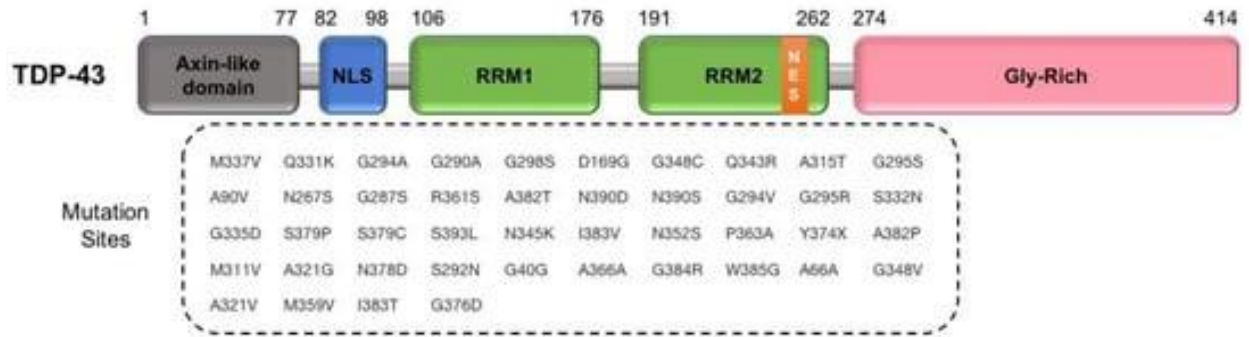


Figure 8 Schematic overview of TDP-43 protein structure (at the top) and the different variant sites in TDP-43. The data were extracted from the ALS online database (ALSoD) from Yousefian-Jazi et al., 2020 and initially from Abel et al., 2012. The figure is from the paper of Yousefian-Jazi et al., 2020

Regarding the pathotoxicity of TDP-43 mutations in ALS aetiology, the concomitant cytoplasmic accumulation of TDP-43 with the loss of nuclear TDP-43 suggest possible GOF and LOF mechanisms.

- GOF hypothesis in TDP-43 ALS pathology is supported by the mislocalisation of TDP-43 in the cytoplasm and thus interactions with new partners. Indeed, TDP-43 dysfunction is known to be associated with mitochondrial stress (W. Wang et al. 2016; Termsarasab et al. 2020), stress granule formation (Baron et al. 2013; Colombrita et al. 2009; Besnard-Guérin 2020), aberrant proteostasis (Casella et al. 2019; D. K. H. Nguyen, Thombre, and Wang 2019), disruption in RNA metabolism and energy metabolism (Weskamp and Barmada 2018; Charif et al. 2020, Scotter, Chen, and Shaw 2015; Shenouda et al. 2018). As to whether the toxicity is mediated by the mutated protein alone or by the formation of aggregates the answer is still not clear. In fact, TDP-43 display a C-terminal region, which is aggregation prone similarly to prion domains (Santamaria et al. 2017). Mutations in this domain were shown to enhance its intrinsic aggregation propensity (B. S. Johnson et al. 2009). With time, mislocalisation and increased TDP-43 aggregates in the cytoplasm lead to aberrations of neuronal proteostasis and overloading of Ubiquitin-proteasome system (UPS), autophagy and endoplasmic reticulum (ER) stress-activated unfolded protein response (UPR) systems (Budini et al. 2017; Ramesh and Pandey 2017). Impaired endocytic systems were also linked with TDP-43 aggregation, supported by co-localisation of the latter with endocytosis-associated proteins in yeast and cellular models (G. Liu et al. 2017).

- With regard to the hypothesis of a LOF, it is mainly attributed to a decrease in the regulation of transcription. TDP-43 was shown to be involved in the splicing of cryptic exons of specific mRNAs, thus regulating their level of transcription. Cryptic exons are referred to splicing variants that may introduce frameshifts or stop codons (Calarco 2013). TDP-43 depletion was shown to cause splicing defects leading to the depletion of genes with long introns (Polymenidou et al. 2011) and emergence of cryptic exons (Humphrey et al. 2017). In fact, several important genes are regulated by TDP-43. For instance, TDP-43 depletion was shown to conduct to loss of stathmin-2 leading to neuronal damage (Klim et al. 2019; Melamed et al. 2019). Stathmin-2 is a gene involved in neurite outgrowth, axonal maintenance, and whose loss accelerates a neuronal degeneration program (Benarroch 2021). Besides the example of stathmin-2, TDP-43 was also shown to be involved in cryptic exon splicing of genes linked to autophagy (Torres et al. 2018), proteostasis (D. K. H. Nguyen, Thombre, and Wang 2019) and certainly many more.

Adding to the complexity of the pathology, the GOF and LOF phenotypes may share similar mechanisms, in particular abnormal splicing. The gain of TDP-43 function could lead to the skipping of constitutive exons, while the loss of function could result in intron retention (Fratta et al. 2018).

Altogether, the toxicity of mutated TDP-43 remains to date not fully elucidated but would seem to be based on a synergic of mechanisms of LOF and GOF. The fact that the Wild-type (WT) protein alone overexpressed or having no NLS signal and therefore expressed only in the cytoplasm, is sufficient to generate neuronal defects, suggests that the WT protein could also contribute to the pathology (Walker et al. 2015). This is also reinforced by the presence in the majority of fALS and sALS cases of TDP-43 positive aggregates.

Fused in sarcoma (FUS)

In 2009, two independent groups identified several mutations in the *fused-in sarcoma/Translocated in Liposarcoma (FUS/TLS)* gene, which segregate with fALS cases (Vance et al. 2009; Kwiatkowski et al. 2009). Rapidly, linkage with sALS was also confirmed (Rademakers et al. 2010; V. V. Belzil et al. 2009). FUS mutations are known to be also the most common cause of juvenile onset ALS. FUS is an ubiquitously expressed DNA and RNA-binding protein (RBP) located predominantly in nucleus, but notable cytoplasmic presence is observed in neurons. Regarding

the structure of the protein, the N terminal is composed of a transcriptional activation domain, followed by a highly conserved RNA recognition motif, multiple 3 glycine-arginine (RGG) rich domains and the C terminal containing the NLS domain (Morohoshi et al. 1998; Svetoni, Frisone, and Paronetto 2016) (Figure 9). FUS protein is involved in various cellular processes, such as nucleo-cytoplasmic shuttling, DNA repair, dendritic RNA transport, cell proliferation, transcription regulation and RNA and microRNA processing and stress granules formation (H. Deng, Gao, and Jankovic 2014; Lagier-Tourenne, Polymenidou, and Cleveland 2010; Ratti and Buratti 2016). In line with the vast implication of FUS in many cellular processes, a recent study using the pull down technique of wild type and mutant FUS followed by a proteomic study identified several interactors and opened new insight in physiological functions of FUS (Kamelgarn et al. 2016). Furthermore, FUS was found to interact with exosomes components, and be itself located in exosomes, suggesting a possible way of cell-to-cell spreading of the pathotoxicity (Kamelgarn et al. 2016).

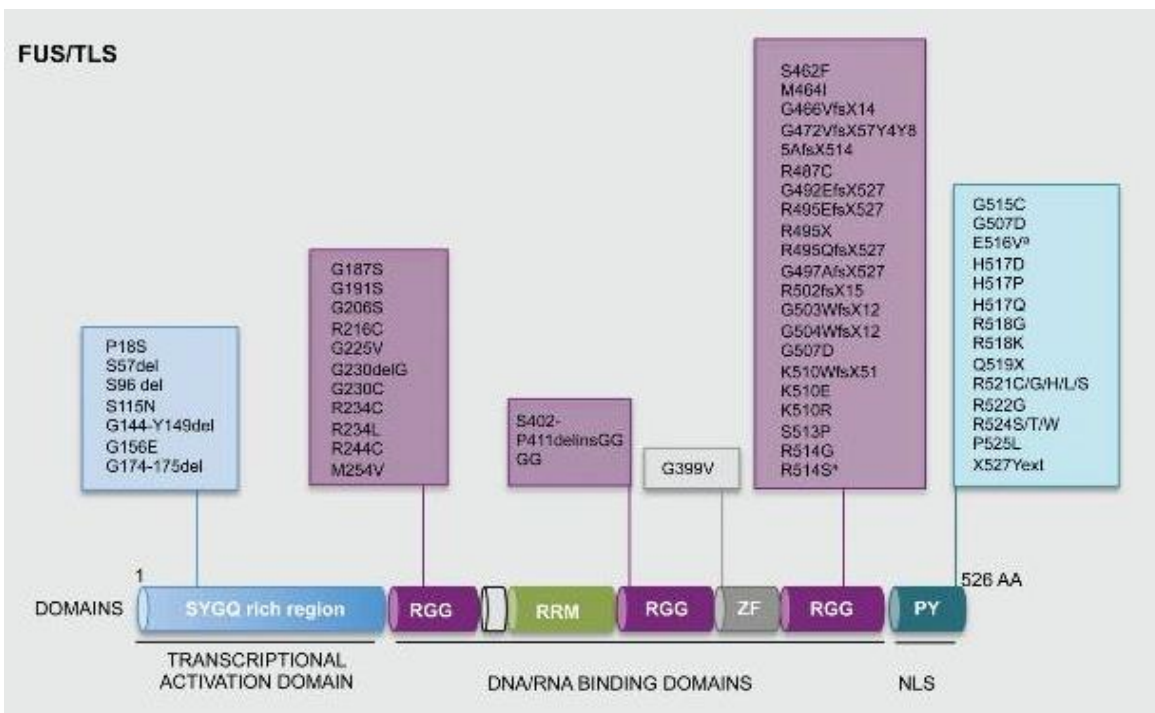


Figure 9 Schematic representation of FUS/TLS sequence. FUS is composed of three major domains at the N terminal the transcriptional activation domain, followed by DNA/RNA binding domains and lastly the NLS domain allowing being addressed in the nucleus. Some most common mutations identified in ALS patients are represented on the figure. The image is extracted from Svetoni et al., 2016 publication.

FUS mutations account for about 4% of fALS and 1% of sALS (Vance et al. 2009; V. V. Belzil et al. 2009; Corrado et al. 2009; Rademakers et al. 2010; H. Deng, Gao, and Jankovic 2014). More than 50 mutations have been identified, most of them being missense dominant mutations, which are predominantly located in two regions of the gene (Figure 9). Around two third of them are located in the exon 12-15 encoding zing-finger motif domain, RGG domains, and the NLS; while the remaining are located in exons 3-6 which encode the N-terminal domain and RGG1 thus affecting RNA binding (H. Deng, Gao, and Jankovic 2014). Mutations found between exons 12-15 tend to cluster more with fALS cases while those found in exons 3-6 are usually associated with sALS. Other mutations found in exons 3-6 do not segregate with the disease, suggesting a potential role as disease modifying factor or incomplete penetrance for these variants (H. Deng, Gao, and Jankovic 2014).

At the clinical level, the site of onset of the pathology is dominantly cervical, with prominent lower MNs degeneration and to lesser extent corticospinal neurons segmentation. *FUS* ALS pathology is associated with mild cognitive impairments (Swinnen and Robberecht 2014).

FUS mutations are similarly to TDP-43 mutations thought to be toxic via a LOF and GOF mechanisms.

- Regarding the GOF hypothesis: cytoplasmic mis-localisation of the protein, accumulation and the formation of stress granule-like structures and other RBP complexes may lead to gain of toxicity (Blokhuys et al. 2013). The stress-granule-like-structure gain of toxicity hypothesis has been well reviewed by Gao et al., 2017 and will not be further discussed herein (F.-B. Gao, Almeida, and Lopez-Gonzalez 2017). Moreover, some other studies also reported GOF mechanisms from mutant *FUS* leading to neuromuscular junctions (NMJ) abnormalities (Sharma et al. 2016), aberrant p38 MAPK activation (Sama et al. 2017) and mitochondrial dysfunction (Tsai et al. 2020).
- The LOF mechanisms are partly related to the depletion of *FUS* from the nucleus and thus causing a partial loss of its nuclear functions (Ishigaki and Sobue 2018). Indeed, alteration of gene expression and or alternative splicing of specific genes could have a major impact on neuronal functions, and contribute to neurodegeneration observed in ALS (Ishigaki and

Sobue 2018). A crosslinking and immunoprecipitation study identified physiological and pathological RNA targets of FUS and thus offer a better insight in the possible genes that are dysregulated in FUS-pathology (Jutzi et al. 2020). Although FUS is enriched in the nucleus, a part of FUS protein is also present in the soma and dendrites of neuronal cells to ensure local translation machinery, transport and mRNA stabilisation (N. Aoki et al. 2012; Yasuda et al. 2013; Qiu et al. 2014; Sephton et al. 2014). FUS binding to the 3' UTR of target mRNA is important for translational efficiency and mRNA stability. Several studies support the implication of FUS in dendritic spines maturation by stabilising several mRNAs (Udagawa et al. 2015; Yokoi et al. 2017). Therefore, a LOF is suggested to lead to destabilised mRNAs (Ishigaki and Sobue 2018) and thus affecting dendritic spine maturation.

Overall, similarly to TDP-43 it is thought that GOF and LOF may share similar mechanisms in the nucleus and may contribute to the pathology (An et al. 2019; Sun et al. 2015).

Altogether, mutant FUS mediated pathomechanisms may be multiple and imply modulations in several key cellular functions. FUS directly affects RNA metabolism via alternative splicing, transcription and mRNA stabilisation. In addition mutant FUS misconformation could confer new properties to the protein and lead to a GOF mechanisms.

Together, since 1993 many genes have been linked to the pathology of ALS, the four major genes which have been described herein are the genes most frequently found mutated in fALS and sALS cases. The study of these genes made it possible to elucidate some shared pathological mechanisms between the different mutations, but also demonstrated the complexity of the pathology. There is not only heterogeneity between variants of the same genes, but also between the different genes involved. The expression of these four genes being ubiquitous, the implication that the coding protein can have within different cell types can be variable. Especially since the pathological mechanisms linked to the mutations seem to be not only a partial LOF, but also an unknown toxic GOF.

Thanks to advances in sequencing technologies, rare ALS linked variants were also identified, and contribute to the better understanding of molecular mechanisms underlying ALS pathology. Interestingly, many recently discovered genes are involved in one way or another in the cellular

degradation machinery (e.g. Tank-Binding kinase 1 (TBK1), Valosine (VCP), Ubiquilin 2 (UBQLN2), Optineurin (OPTN), Sequestosome 1 (SQSTM1), Charged Multivesicular Body Protein 2B (CHMP2B)), and some of them are also linked to immune functions (e.g. TBK1). Although these variants represent a very small proportion of ALS cases, they open up new perspectives to highlight on possible pathomechanisms common to the different ALS forms.

1.3 Environmental risk factors

That said, despite a genetic predisposition that may exist in some ALS cases, the fact that the majority of cases are sporadic suggest that other external factors might play a role of risk factors. To date, the most consistent risk factors remain age, sex, as well as a family history of the disease (Kurtzke 1991; Worms 2001; Armon 2003). However, some studies have highlighted certain external factors that could contribute to the risk of developing ALS.

Earliest environmental factor reported was beta Methylanmino-L-Alanine (BMAA) exposure, which is a neurotoxin, found in seeds of cycads. This neurotoxin was thought to enter human food chain as cycads seeds were directly consumed by humans or by wild or feral animals, which were subsequently eaten by humans. The earliest reports were made in Chamorro people of Guam Island as cycads seeds were prominently consumed by this ethny and increased incidence of 50-100 times the ALS incidence (Kurland and Mulder 1954). Since then other environmental risk factors have been suggested such as repeated head injury and physical exercise (A. Chiò et al. 2009; Savica et al. 2012; Pupillo et al. 2012; Veldink et al. 2005), heavy metal exposure (Kamel et al. 2003; 2002; F. Fang et al. 2010; Garzillo et al. 2014; Garuda et al. 2019), smoking, drinking (Armon 2009; Alonso, Logroscino, and Hernán 2010; Gallo et al. 2009; de Jong et al. 2012; Lian et al. 2019, 201) and the dietary which suggested that antioxidant based diets seem to be protective factor for ALS (H. Wang et al. 2011; Veldink et al. 2007; Fitzgerald et al. 2014; Kazushi Okamoto et al. 2007). The activity have also been reported as a potential risk factor for developing ALS, mainly because increased rates of ALS were reported in US military veterans (Beard and Kamel 2015). However, as the militaries can be exposed to a myriad of harmful factors ranging from psychological trauma to specific virus or environmental toxicants specific to the area where they are deployed, it is difficult to delineate the exact risk factors. Studies suggested to be a combination of various different factors notably those mentioned above such as smoking, heavy

metal exposure, repeated trauma or else. Further studies would be needed to confirm these associations.

In conclusion, ALS appears to be a multifactorial disease. Genetic predispositions are certainly risk factors but it seems that other external factors can strongly contribute to the risk of developing ALS. Especially since the majority of cases are sporadic, and that different ALS phenotypes exist. More comprehensive epidemiological studies should be carried out in order to better understand the role of external factors to the contribution of the disease.

2- Pathological hallmarks in ALS

In this chapter, I will present some pathological hallmarks observed in ALS patients, which are found in MNs or in surrounding glial cells. In a first part I will present the signatures observed in MNs, which are either shared or distinct in different ALS subtypes. In a second part I will detail evidences of neuroinflammation in ALS as a hallmark of the disease, firstly based on post mortem studies, but also across disease progression with brain imaging studies as well as biofluids circulating inflammatory markers.

2.1 Motor neuronal suffering and degeneration

Amongst neuronal populations, MNs have a unique feature as they display a large soma and a very long axon. In ALS, the neurodegenerative mechanisms leading to MNs loss are not fully understood. Indeed, it remains elusive why MNs are the main neuronal population targeted while proteins linked genetically or pathologically with ALS are expressed ubiquitously. Moreover, not all MN subpopulations are affected equally (Ragagnin et al. 2019). Corticospinal MNs and spinal MNs innervating voluntary muscles are more prone to degeneration compared to other subtypes of MNs. While oculomotor and Onuf nucleus MNs are the latest ones to degenerate (Obál, Engelhardt, and Siklós 2006).

2.1.1 MNs degeneration time frame

ALS pathology is thought to be compensatory for a long period before symptoms appear (Fischer et al. 2004; Rocha et al. 2013; Clark et al. 2016). From animal studies and very rare human studies whom died at early phase of the pathology, some hypotheses regarding the timeframe of pathological events were suggested. Initially, MN degeneration is compensated by sprouting and by projecting collaterals of neighbouring subtypes of MNs, which are less vulnerable to degeneration (Carvalho and Swash 2013). At this stage, degenerated MNs do not present abnormal soma, however they present denervated NMJs (Fischer et al. 2004; Rocha et al. 2013; Clark et al. 2016). As disease progresses, other MNs are becoming affected and lose the capacity to effectively compensate (Swinnen and Robberecht 2014). There are hypotheses, supporting a dying back mechanism of MNs, meaning that the pathology would spread from periphery to CNS. In parallel, a dying forward hypothesis is also proposed suggesting that the pathology spreads from the CNS to the periphery. These hypothesis are mainly based on hyperactivity of cortical neurons and defects in axonal transports (Baker 2014; Shibuya 2017; Eisen 2021).

2.1.2 Post-mortem observations

Post-mortem ALS patients studies at a late stage of the pathology present rarefaction of Betz cells in the cortex and MNs in ventral horns of the spinal cord accompanied by increased microglial and astrocytic activation (Brownell, Oppenheimer, and Hughes 1970; Nihei, McKee, and KowaU, n.d. 1993; Kawamata et al. 1992; Saberi et al. 2015). Interestingly sensory neurons of the spinal cord dorsal horns do not degenerate, and do not present microglial activation, strongly suggesting the specificity of the microgliosis and astrogliosis in reaction to degenerating neurons (Review Liu et al., 2021). Remaining cells' soma are shrunk and display inclusions containing aggregated proteins (which will be discussed below). At the periphery, nerve demyelination has been observed accompanied by axonal degeneration, abnormal motor end plates at the NMJ, axonal sprouting linked with tentative to reinnervate muscles, and atrophic muscles (Bjornskov, Norris, and Mower-Kuby 1984; Tandan and Bradley 1985).

2.1.3 Protein aggregates as pathological markers

Protein aggregates are pathological hallmarks of multiple neurodegenerative diseases, so are they in ALS pathology. Although their presence in affected MNs is characteristic, their role in ALS

disease is still poorly understood, and at the moment we do not know if these aggregates are drivers, consequences or both in the pathology. Nonetheless, it seems that the aggregates are the consequence of misfolded proteins which can be linked to ALS mutations, protein damages or else. In most cases, proteins encoded by ALS linked-genes are present in aggregates in mutation carriers as well as a large proportion of non-mutation carriers, indicating a more widespread role of these aggregates in the pathology. Additionally in some cases, some of these proteins are also present in other ND such as FTD and AD suggesting a wider implication of the proteins aggregated in neurodegeneration (Sami et al. 2017).

Characteristics inclusions

In ALS patients post-mortem tissues different types of aggregates are found. Ubiquitinated inclusions can be classified as Skein-like inclusions (SLI), Lewy body-like hyaline inclusions (LBHI) or Round hyaline inclusions (RHI) (Blokhuis et al. 2013). SLI are the specific hallmark of ALS, while other types of inclusions can also be found in other disorders (Bendotti et al. 2012). At the ultrastructure level, SLI appear with randomly oriented filaments covered by fine granules. They may result from ubiquitin aggregation to another protein abnormally accumulated within the cytoplasm, and often colocalised with the SQSTM1/p62 an autophagosome cargo protein involved in selective autophagy (van Welsem et al. 2002; Mizuno et al. 2006). These types of inclusions are usually labelled using anti-ubiquitin antibodies. Ubiquitin positive inclusions are found in degenerating MNs but also in normal morphology MNs suggesting that it could be a protective mechanism before MN death. A significant positive correlation was found between the severity of neuronal loss and the proportion of SLI positive neurons in the spinal cord of sALS (van Welsem et al. 2002).

The other commonly found aggregates and which are also specific to ALS pathology, are Bunina bodies, which are small eosinophilic ubiquitin-negative inclusions. On electron microscopy Bunina bodies consist of amorphous electron-dense material surrounded by tubular and vesicular structures (Koichi Okamoto, Mizuno, and Fujita 2008). Bunina bodies are usually immunonegative for TDP-43 and immunopositive for cystatin C, transferrin, peripherin and Sortilin-related receptor CNS expressed 2 (SorCS2) (Koichi Okamoto et al. 1993; Mizuno et al. 2006; 2011; Mori et al. 2015; Miki et al. 2018).

Furthermore, Neurofilament (NF) accumulation in MNs are observed in early ALS in all ALS forms. They appear as axonal spheroids in lower MNs and conglomerates in upper MNs (S. M. Chou, Wang, and Komai 1996; Mendonça, Chimelli, and Martinez 2005). NF are the major structural elements of the neuronal cytoskeleton. They are composed of three different polypeptides which are light (NF-L), medium (NF-M) and heavy (NF-H) chains. In addition, NF accumulation is also observed in other ND such as AD or PD. However, the exact role of these aggregates in the different pathologies are to date still under investigations (Didonna and Opal 2019). Overall, although most of the descriptions of inclusions were reported in neuronal cells, the presence of aggregates in glial cells has also been observed (Miller, Cookson, and Dickson 2004). Circulating NF were proposed as biomarkers for disease progression in ALS (Poesen et al. 2017; Benatar et al. 2018)

TDP-43 inclusions

A breakthrough was achieved when the wt-TDP-43 protein was identified as a major component of ubiquitinated inclusions in ALS (Arai et al. 2006; M. Neumann et al. 2006). Non-mutated ubiquitinated or phosphorylated TDP-43 deposits were observed in almost all sALS studied cases and some fALS, except in SOD1 mutated ALS patients (Mackenzie et al. 2007; Smethurst, Sidle, and Hardy 2015; Miki et al. 2018). Spinal cord MNs contain TDP-43 positive inclusions, which appear in different cell compartments and under different forms such as : cytoplasmic with compact rounded or skein-like appearance; in neurites with short or long dystrophic morphologies or intranuclear (Van Deerlin et al. 2008). Inclusions were also observed in neurons of the frontal cortex, pre-frontal gyrus as well as in neighbouring glial cells (Mackenzie et al. 2007; Tan et al. 2007; H. Zhang et al. 2008). More recently, TDP-43 was shown to be associated with Bunina bodies in some sALS and co-localised with some Bunina bodies related proteins (Cystatin C, transferrin, SorCS2), suggesting a relationship between Bunina bodies and TDP-43 (Miki et al. 2018). In fact, it has been suggested that severity of MNs degeneration may be proportional to aggregated TDP-43 levels (Brettschneider et al. 2013; 2014). TDP-43 inclusion's presence in almost all ALS cases, as well as other ND, suggests a prominent role in neurodegenerative pathologies. Interestingly, Cykowski and collaborators tried to understand the clinical significance of TDP-43 pathology. They observed a positive correlation between TDP-43 pathology burden and cognitive impairment, though no correlation was made with disease duration or progression rate (Cykowski et al. 2017). Furthermore, the Hergesheimer and collaborators' extensive review depicted the central role of

TDP-43 aggregates in neurodegeneration observed in ALS. They also put in perspective the possibly interesting therapeutic intervention that could be the clearing TDP-43 aggregates during the early stages of the disease as it is a shared pathological hallmark in almost all ALS patients (Hergesheimer et al. 2019).

SOD1 inclusions

In SOD1 mutated patients, skein-like cytoplasmic inclusions have been observed. Usually these inclusions are TDP-43 and FUS immuno-negative (Nakamura et al. 2014). Ubiquitinated mutant SOD1 protein is a hallmark of a subset of fALS patients carrying mutations in this gene, while misfolded wt-SOD1 aggregates have been found more widely in sporadic patients. In fact, oxidised wt-SOD1 was shown to be prone to misfolding, and subsequently aggregation. Some studies using conformation specific SOD1 antibodies, observed small round inclusions in the spinal cord of sALS and non-SOD1 mutation carriers fALS (Forsberg et al. 2010; Bosco et al. 2010; Paré et al. 2018; Medinas et al. 2018). These inclusions were usually located in soma and axon hillock. Small granular inclusions were also observed in the high majority of these sALS and non-SOD1 carrier fALS, which partially co-localised with lysosomal marker Cathepsin D (Forsberg et al. 2010). Most of the controls lacked SOD1 granular staining. Taken together, this suggests a possible shared SOD1 dependent pathogenic pathway between mutant SOD1 fALS and sALS.

RNA foci and C9orf72 linked DPR

At the neuropathological level, C9orf72 mutated ALS and FTD patients present TDP-43 positive inclusions in neurons and glia, and high proportion of nuclear RNA foci in frontal cortex and spinal cord neurons as well as ubiquitin positive inclusions. C9orf72 RNAs containing either sense G4C2 or antisense C4G2 repeats with uncharacteristic secondary structures are thought to generate RNA foci and interact with RBP. Both sense and antisense RNAs foci were observed in multiple CNS regions of C9-ALS patients' brains (Cooper-Knock et al. 2014; DeJesus-Hernandez et al. 2011). In general, RNA foci are more abundantly found in neurons but can also be found in astrocytes, oligodendrocytes and microglia at lower frequencies (Gendron et al. 2013; Lagier-Tourenne et al. 2013; Mizielinska et al. 2013). Most RNA foci are located within the nucleus but cytoplasmic RNA foci were also observed (Mizielinska et al. 2013; Y.-B. Lee et al. 2013; Cooper-Knock et al. 2015; Swinnen et al. 2018). Regarding DPR, proteins translated from the sense RNA repeats, including

GA, GP, and GR, are more commonly found in ALS patients compared to DPR translated from antisense RNA repeats, including PR and PA (Gendron et al. 2013).

FUS inclusions

Pathological examinations of post-mortem tissues of FUS mutation carriers' show FUS positive cytoplasmic inclusions in MNs of the anterior horns. Additionally, FUS-positive inclusions were also observed in glial cells (Suzuki et al. 2012). FUS positive inclusions were found as large globular and elongated cytoplasmic inclusion (Vance et al. 2009; M. Neumann et al. 2009; E. J. Huang et al. 2010). Few studies reported some FUS-positive inclusions in sALS and fALS patients, except for SOD1 mutation carriers (H.-X. Deng et al. 2010; Tyzack et al. 2019). Moreover, FUS aggregates are also found in other ND such as FTD or Neuronal intranuclear inclusion disease (NIID) (M. Neumann et al. 2009; Doi et al. 2010; Woulfe, Gray, and Mackenzie 2010), suggesting a wider implication in neurodegeneration processes. In FUS mutated patients, TDP-43 was normally distributed in the cell and positive inclusions in the cytoplasm and nucleus of neurons and glial cells were also absent, thereby distinguishing FUS mutation carriers from other ALS cases.

Overall, both upper and lower MNs degenerations are a prominent hallmark of ALS pathology although soma rarefaction is predominant in the spinal cord. Presence of aggregates in affected cells is a dominant signature of many ND, with no exception in ALS. TDP-43 are the most common signature between the different ALS forms, being present in almost 95% of cases. Other types of aggregates are also present which are more ALS subtypes specific, such as misfolded mutant SOD1, FUS, RNA foci and DPR. Nonetheless, post-mortem studies represent a late stage of the pathology and it is therefore difficult to identify the mechanisms possibly responsible for these phenotypes. Animal and cellular models have been of great help to identify early disease signatures in MNs cells (see below).

2.2 Neuroinflammation as a pathological hallmark of ALS disease

Neuroinflammation is a multi-step physiological response within the brain or spinal cord, triggered by cellular damages or infectious pathogens infiltrating the brain parenchyma. The reaction is orchestrated by local innate immune glial cells, endothelial cells, and peripheral immune cells (Figure 10). The inflammatory event is mediated by numerous circulating factors such as cytokines,

chemokines, reactive oxygen species (ROS) and secondary messengers (DiSabato, Quan, and Godbout 2016). These factors induce biochemical cascades, which contribute to the remodelling of the CNS micro-environment (Glass et al. 2010; DiSabato, Quan, and Godbout 2016). Primarily, neuroinflammation is a physiological response, which aims at restoring brain homeostasis; however, depending on the intensity and duration of the inflammation, the response can turn from supportive to destructive to the CNS (González et al. 2014; DiSabato, Quan, and Godbout 2016). As a matter of fact, the inflammation response represents a two edged sword needing strict regulation, as an excessive or deficient reaction might lead to pathological conditions (Sochocka, Diniz, and Leszek 2017).

2.2.1 The different immune systems

Two immune systems interplay to govern inflammatory events (Figure 10).

- The innate immune system, which is the first line of defense of the organism, provides immediate immune response. It encompasses a group of different cell types, such as microglia, tissue resident macrophages, dendritic cells, harbouring various functions in order to mediate clearance of external pathogens, cellular debris, or other insults for the organism. These cells are distributed throughout the body and their functions may depend on their microenvironment (e.g. microglia in the brain, macrophages in different tissues).
- The adaptive immune system, which is highly specific to particular pathogens that can induce it and provide long lasting immune protection. Major actors of the adaptive immune system are T and B cells, which can be further divided in many subclasses and bear distinct roles during inflammatory events (review Liu et al., 2021).

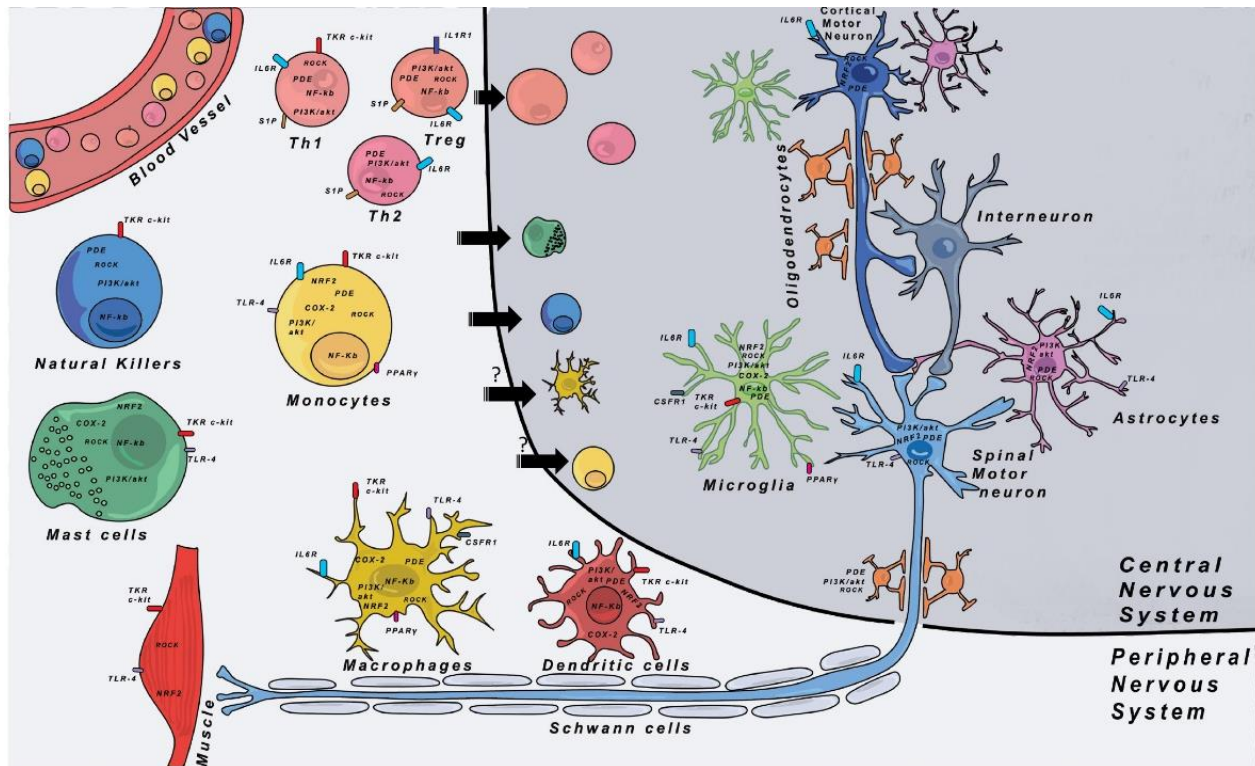


Figure 10 A simplified schema identifying the main Immune cells and their respective locations in the CNS and PNS, as well as other cells in the CNS supporting MNs. In the CNS, under homeostatic condition, the major innate immune cells of the brain parenchyma are microglia cells, while on extraparenchymal regions are present other brain macrophages (reviewed by Brioschi et al., 2020). In pathological condition, some cells can infiltrate the brain parenchyma (indicated by the black arrows) such as mast cells, natural killers, T cells, monocytes and possibly others. In the PNS, the innate immune cells are macrophages, monocytes, dendritic cells, mast cells and natural killers; adaptive immune cells are T and B cells. In pathological condition, peripheral inflammation lead to the recruitment of these different cell types at the site of injury in order to resolve local inflammation and injury

2.2.2 ALS patients post mortem tissues' examination

For some time, research has focused on neuroinflammation as a major component of neurodegenerative diseases. It is now well accepted as a prominent hallmark of many ND, ALS is no exception (W.-W. Chen, Zhang, and Huang 2016). In ALS, first observations came from post-mortem studies of ALS patients. As mentioned above, microgliosis and astrogliosis are prominent signatures observed in the cortex and spinal cord surrounding degenerating neuronal cells (Brownell, Oppenheimer, and Hughes 1970; T. Kawamata et al. 1992a; Brettschneider et al. 2012; Saber et al. 2015). Since then, studies have shown presence of activated CD68+ macrophages/microglia, granular or degranulating mast cells, and of CD4+ and CD8+ lymphocytes in the vicinity of MNs (T. Kawamata et al. 1992a; Henkel et al. 2004; Kovacs et al. 2021).

Nevertheless, as post-mortem studies are covering a late phase of the pathology, it is difficult to know what is the role of neuroinflammation during the course of the pathology.

2.2.3 In vivo observation of ALS neuroinflammation using imaging technology

Brain imaging studies have been a valuable tool to image changes in cell reactions, the pattern of spreading of the disease and inflammation during the disease progression *in vivo*. Such imaging studies are now even investigated as biomarkers for early detection of ALS phenocconversion, corresponding to the transition from the pre-symptomatic phase to the symptomatic phase (S. Chew and Atassi 2019). Positron Emission Tomography (PET), a subtype of imaging techniques, has been widely used to measure changes in metabolic processes. Translocator protein (TSPO) is a 18kD outer mitochondrial membrane protein highly expressed on activated microglia and astrocytes (A. K. Brown et al. 2007; Lavisse et al. 2012), as it has important functions in immune reactions such as redox or calcium homeostasis (Gatliff et al. 2014; Guilarte, Loth, and Guariglia 2016; Gatliff et al. 2017). TSPO is used as a target to study inflammatory reactions. Several generations of radioligands succeeded after the discovery of TSPO polymorphism in the population, which explains the observed inter-individual variability in radioligands binding affinity with TSPO (Owen et al. 2012; K. Kim et al. 2020).

Sundry of studies using the most commonly used radiomarker [11C]-PBR28 have shown an increased signal in the cortical and subcortical motor regions (e.g. bilateral motor cortices, supplementary motor area, upper region of the corticospinal tract) of ALS patients compared to controls (Zürcher et al. 2015; Alshikho et al. 2016; 2018). Magnetic resonance scanner in parallel to PET scanning, brought insight into structural abnormalities, and revealed correlation and colocalisation between increased [11C]-PBR28 uptake and cortical thinning in the region (Alshikho et al. 2016). In addition to structural abnormalities, the [11C]-PBR28 signals' strength in the precentral gyrus was also correlated with upper motor neuron disease burden (UMNB) (Zürcher et al. 2015; Alshikho et al. 2018). Interestingly, the site of onset appeared also to correlate with inflammation; in fact, Zürcher et al showed in their study that patients with onset at the spinal level had an increased signal in the precentral gyrus region, while those with bulbar onset had an increased signal in the brainstem (Zürcher et al. 2015). More recently, multimodal molecular neuroimaging tools were developed to evaluate the relationship between several brain metabolites and thus correlated underlying pathomechanisms. In fact, the latest study from Ratai

and collaborators correlated inflammatory events with neuronal/axonal injury/loss in the precentral gyri of ALS patients, while it was not observed in other brain regions (Ratai et al. 2018).

The majority of imaging studies in patients are cerebral. In spite of numerous attempts to image the spinal cord in vivo (Bede et al. 2012) technological constraints (i.e. respiration, cardiac movements, small cross-sectional area) have precluded reliable quantitative spinal cord imaging in patients (El Mendili et al. 2019). Amongst the scarce imaging studies assessing ALS patient's spinal cords, most of them used magnetic resonance imaging techniques. Studies of metabolic changes in ALS patients' spinal cords using whole body PET/computed tomography (CT) images are recent. Two independent studies identified increased [18F]-FDG uptake in the spinal cord while it was decreased in the brain motor cortex. [18F]-FDG is thought to reflect cell metabolism without specificity for glial cells, but the observed hyper-metabolism in the spinal cord was suggested to represent an increased inflammation and gliosis due to accumulating glial cells in reaction to degenerating neuronal cells (Bauckneht et al. 2020). Further studies using specific radioligands will have to confirm these observations.

Taken together, imaging studies are valuable tools to study upper MNs dysfunctions and neuroinflammatory events in vivo at spatial and temporal levels. Imaging of the spinal cord is still recent, but will help to further elucidate the inflammatory events in the spinal cord of ALS patients. That said, TSPO is expressed in both astrocytes and microglia, given their different roles in ALS pathology, it implies a significant limitation in data interpretations (Vivash and O'Brien 2016). [18F]-DPA-714 radioligand have previously demonstrated higher specificity of TSPO and was suggested to better detect microglial cells activation, yet it is still poorly used in routine (Corcia et al. 2012; Golla et al. 2015; Hamelin et al. 2016; Coda et al. 2021; Rodríguez-Chinchilla et al. 2020). Further studies with microglial specific radioligands would be warranted to strengthen the observations and subsequent conclusions.

2.2.4 Circulating inflammatory markers in ALS patients

The presence of inflammatory markers in biological fluids is widely studied in ALS patients and allows monitoring of inflammatory events in the CNS and PNS. A large panel of cytokines and

chemokines have been identified in the cerebrospinal fluid (CSF) and blood (Lu et al. 2016; Michaelson et al. 2017) (Table 2, Table 3, Table 4).

- Interleukins are a group of cytokines, which are in majority synthesised by helper CD4 T lymphocytes, monocytes, macrophages and endothelial cells. They are involved in many cellular functions and contribute to pro- and anti-inflammatory reactions. The most consistently found deregulated cytokines are IL-2, IL-6, IL-8, IL-10, IL-15 and IL-17 (). In most studies, interleukins were found increased in ALS patients compared to controls. Nevertheless, some cytokines circulating levels remain controversial. For instance, IL-10's levels are found to be increased in some studies (Furukawa et al. 2015; Lu et al. 2016; J. Guo et al. 2017) while in others the levels were found to be decreased (R. M. Mitchell et al. 2009). Interleukins serve as activators of the innate and adaptive immune systems. Their altered levels observed in ALS patients in comparison to controls reflect an ongoing inflammatory event, without knowing which cells exactly are secreting these factors (review Liu et al., 2021). Some studies reported correlation between the level of interleukins and disease progression (IL-4, IL-10, IL-6). For example, IL-2 was found to be increased in most studies. However, it was found decreased in one study at the second visit of the patient, which may represent a more tardive phase of the pathology. This cytokine plays a central role in the adaptive immune system of ALS patients, as it has a major role in activation, expansion and differentiation of T cell subsets. It acts directly on Th1/Th17 expansion and Treg/Th2 proliferation. Thus, the early increase in IL-2 may reflect a phase in which the organism is combating against the pathology with a balance between Th1/Th17 and Treg/Th2. While the decrease was found at a later stage, corroborating findings of decreased Tregs numbers in ALS patients (Beers, Zhao, and Appel 2018). A clinical trial, which infuses low dose IL-2 in order to force production of Tregs by ALS patients, has been launched recently and will be further discussed in the next part. This example of IL-2 variability shows the importance in the temporality of secretion of different interleukins, which may play different roles throughout the disease.

Table 2 (Part I) Circulating interleukins in ALS patients' biofluids. Disease duration is expressed in mean/SEM or SD; mean/ range ; PR/ SEM in months. Tp: Timepoint

| Name | Biofluids | No of patients | ALS form | Disease duration (mo) | 2 ^d TP | Comparison ALS to Ctrl | Associations | References | |
|--------------|-------------|----------------|---------------------|-----------------------------|--|---------------------------|---|---|-----------------------------|
| Interleukins | IL-6 | 39 | sALS | 16.6 (± 2.4) | | ↗ | | Mitchell et al., 2009 | |
| | | 2 | fALS | Undefined | | ↗ | | Mitchell et al., 2009 | |
| | | plasma | 68 | Undefined | 36 (± 32.4) | n=24 6.8 mo (5.6–11.5) | ↗ | No differences at the follow up | Prado et al., 2018 |
| | | Plasma | 95 | Undefined | 22.4 (12.5–32.2) | | ↗ | Patients with ALS at earlier disease stage lower IL-6 | Lu et al., 2016 |
| | IL-6 | CSF | 77 | Undefined | Undefined | | ↗ | | Gonzalez-Garza et al., 2018 |
| | | CSF + serum | 105 | sALS | 30.70 (± 29.70) | | ↗ | | Guo et al., 2017 |
| | | CSF | 26 | sALS | 14.5 (± 10.9) | | ↗ | | Furukawa et al., 2015 |
| | | Serum | 10 | sALS | PR: 1.1 (± 0.2) | | ↗ | | Blasco et al., 2016 |
| | | CSF + serum | 20 | sALS hypoxemic / normoxemic | B (#11): 19.6 (± 7.6) S (#9): 17.6 (± 6.92) | | ↗ | | Moreau et al., 2005 |
| | IL-10 | Serum | 13 | 11sALS 2 fALS | 21.0 (± 4.44) | n=13 6mo | ↗ | Second visit no differences to controls | Ehrhart et al., 2015 |
| | | Plasma | 79 | Undefined | 21.37 (3.50–137.47) | | ↗ | Pos correlation with age Neg correlation with DPR | Tortelli et al., 2020 |
| | | CSF | 39 | sALS | 16.6 (± 2.4) | | ↗ | Pos correlation with DD Neg correlated with DPR | Mitchell et al., 2009 |
| | | Plasma | 95 | Undefined | 22.4 (12.5–32.2) | | ↗ | | Lu et al., 2016 |
| | | CSF | 2 | fALS | Undefined | | ↗ | | Mitchell et al., 2009 |
| | IL-10 | CSF + serum | 105 | sALS | 30.70 (± 29.70) | | ↗ | | Guo et al., 2017 |
| CSF + plasma | | 164 | 43 fALS 121 sALS | 39.8 (9.4–227) | | ↗ | ↗ only in CSF plasma IL-10 | Olesen et al., 2020 | |
| CSF | | 26 | sALS | 14.5 (± 10.9) | | ↗ | ↗ in ALS compared to MMN | Furukawa et al., 2015 | |
| Plasma | | 79 | Undefined | 21.37 (3.50–137.47) | | ↗ | | Tortelli et al., 2020 | |
| Plasma | | 95 | Undefined | 22.4 (12.5–32.2) | | ↗ | | Lu et al., 2016 | |
| IL-8 | plasma | 68 | Undefined | 36 (± 32.4) | n=24 6.8 mo (5.6–11.5) | ↗ | No differences at the follow up | Prado et al., 2018 | |
| | CSF + serum | 20 | Undefined | 9 (± 4.4) | | ↗ | | Kuhle et al., 2009 | |
| | CSF | 42 | sALS | 13.0 (± 9.3) | | ↗ | | Tateishi et al., 2010 | |
| | CSF | 26 | sALS | 14.5 (± 10.9) | | ↗ | | Furukawa et al., 2015 | |
| | CSF | 77 | Undefined | Undefined | | ↗ | ↗ in ALS compared to MMN | Gonzalez-Garza et al., 2018 | |
| IL-8 | Serum | 10 | sALS | PR: 1.1 (± 0.2) | | ↗ | | Blasco et al., 2017 | |
| | Serum | 13 | 11 sALS 2 fALS | 21.0 ± 4.44 | n=13 6 mo | ↗ | ↗ only at the 2d visit compared to controls | Ehrhart et al., 2015 | |

Table 2 (Part II) Circulating interleukins in ALS patients' biofluids. Disease duration is expressed in mean/SEM or SD; mean/ range ; PR/ SEM in months. Tp: Timepoint

| Name | Biofluids | No of patients | ALS form | Disease duration (mo) | | 2 ^o TP | Comparison ALS to Ctrls | Associations | References |
|--------------|--------------|----------------|-------------------|--|---------------|-------------------|--------------------------|--|-----------------------|
| | | | | ALS | FA | | | | |
| Interleukins | CSF | 39 | sALS | 16.6 (± 2.4) | | | ↗ | | Mitchell et al., 2009 |
| | Plasma | 95 | Undefined | 22.4 (12.5–32.2) | | | ↗ | | Lu et al., 2016 |
| | CSF | 2 | fALS | Undefined | | | ↗ | | Mitchell et al., 2009 |
| | CSF + serum | 105 | sALS | 30.70 (± 29.70) | | | ↗ only in CSF | | Guo et al., 2017 |
| | Serum | 13 | 11 sALS 2 fALS | 21.0 (± 4.44) | n= 13 6 mo | | ↘ | | Ehrhart et al., 2015 |
| | Plasma | 79 | Undefined | 21.37 (3.50–137.47) | | | ↗ | | Tortelli et al., 2020 |
| | CSF | 39 | sALS | 16.6 (± 2.4) | | | ↗ | | Mitchell et al., 2009 |
| | CSF | 2 | fALS | Undefined | | | ↗ | | Mitchell et al., 2009 |
| | CSF + serum | 105 | sALS | 30.70 (± 29.70) | | | ↗ | | Guo et al., 2017 |
| | CSF | 42 | sALS | 13.0 (± 9.3) | | | ↗ | | Tateishi et al., 2010 |
| IL-7 | CSF | 26 | sALS | 14.5 (± 10.9) | | | ↗ in ALS compared to OND | | Furukawa et al., 2015 |
| IL-4 | Plasma | 95 | Undefined | 22.4 (12.5–32.2) | | | ↗ | | Lu et al., 2016 |
| IL-4 | CSF | 26 | sALS | 14.5 (± 10.9) | | | ↗ in ALS compared to MMN | ↗ was correlated with slower disease progression | Furukawa et al., 2015 |
| IL-9 | CSF | 42 | sALS | 13.0 (± 9.3) | | | ↗ | | Tateishi et al., 2010 |
| IL-17 | CSF | 39 | sALS | 16.6 (± 2.4) | | | ↗ | | Mitchell et al., 2009 |
| | CSF | 2 | fALS | Undefined | | | ↗ | | Mitchell et al., 2009 |
| | CSF | 42 | sALS | 13.0 (± 9.3) | | | ↗ | | Tateishi et al., 2010 |
| | CSF + serum | 105 | sALS | 30.70 (± 29.70) | | | ↗ | | Guo et al., 2017 |
| | CSF | 26 | sALS | 14.5 (± 10.9) | | | ↗ in ALS compared to MMN | | Furukawa et al., 2015 |
| IL-17A | Serum | 32 | 28 sALS 4 fALS | sALS 24.5 (± 3.0) fALS 16.0 (± 4.2) | | | ↗ | | Fiala et al., 2010 |
| IL-1b | Plasma | 95 | Undefined | 22.4 (12.5–32.2) | | | ↗ | | Lu et al., 2016 |
| | CSF | 42 | sALS | 13.0 (± 9.3) | | | ↗ | | Tateishi et al., 2010 |
| | CSF + plasma | 164 | 43 fALS 121 sALS | 39.8 (9.4–227) | | | ↗ in plasma | correlated with shorter survival | Olesen et al., 2020 |

Table 2 (Part III) Circulating interleukins in ALS patients' biofluids. Disease duration is expressed in mean/SEM or SD; mean/ range ; PR/ SEM in months. Tp: Timepoint

| Name | | Biofluids | No of patients | ALS form | Disease duration (mo) | 2 ^o TP | Comparison ALS to Ctrlis | Associations | References | |
|---------------------|--|-----------|----------------|----------|-----------------------|-------------------|--------------------------------|--------------------------------|---|-----------------------|
| Interleukins | | IL-18 | 54 & 125 | sALS | 11.32 (1-48) | | ↗ in the serum of ALS patients | | Italiani et al., 2014 | |
| | | | CSF + serum | | | | | | | |
| | | | CSF + plasma | | | | every 4 mo for 2y | ↗ only CSF | ↗ in all, fast progressor and C9 patients | Huang et al., 2020 |
| | | IL-18 BP | CSF + serum | 54 & 125 | sALS | 11.32 (1-48) | | ↗ in the serum of ALS patients | | Italiani et al., 2014 |
| | | IL-12p70 | Plasma | 95 | Undefined | 22.4 (12.5-32.2) | | ↗ | | Lu et al., 2016 |
| | | | CSF | 42 | sALS | 13.0 (± 9.3) | | ↗ | | Tateishi et al., 2010 |
| | | | Plasma | 95 | Undefined | 22.4 (12.5-32.2) | | ↗ | | Lu et al., 2016 |
| | | IL-5 | Serum | 13 | 11 sALS 2 fALS | 21.0 (± 4.44) | n=13 | ↘ | Second visit no differences to controls | Ehrhart et al., 2015 |
| | | IL-13 | Plasma | 95 | Undefined | 22.4 (12.5-32.2) | 6 mo | ↗ | | Lu et al., 2016 |

- ❖ Chemokines are a family of small cytokines or signalling proteins, which are secreted by cells to act on neighbouring cells and induce chemotaxis. They can either be pro-inflammatory in order to recruit necessary cells at the site of injury or homeostatic to control cell migration during physiological processes such as tissue maintenance and development (Laing and Secombes 2004). In ALS blood and/or CSF Monocyte chemoattractant protein 1 (MCP-1), Macrophage inflammatory protein-1 alpha (MIP-1 α /CCL3), Macrophage inflammatory protein-1 β (MIP-1 β), RANTES, Eotaxin-1, C-X-C motif chemokine 11 (CXCL11) and C-X-C motif chemokine ligand 10 (CXCL10) levels were found deregulated compared to controls. The level of MCP-1 correlated with shorter survival and negatively correlated with the ALSFRS-R score (Kuhle et al. 2009; Tateishi et al. 2010). Conversely, MIP-1 α and MIP-1 β levels positively correlate with disease duration and ALSFR-S (Tateishi et al. 2010; X. Yang et al. 2016; J. Guo et al. 2017). A recent study shows the importance of chemoattractant agents in cell recruitment during the disease. In fact, NK cells infiltration in CNS affected regions were observed in sALS and mutant SOD1 mice models. The recruitment in the spinal cord of these NK cells was driven by a peak of MCP-1, and neutralisation of the latter reduces NK infiltration that were found deleterious in the pathology (Garofalo et al. 2020).

Table 3 Circulating chemokines in ALS patients' biofluids. Disease duration is expressed in mean/SEM or SD; mean/range ; PR/ SEM in months. Tp: Timepoint

| Name | Biofluids | No of patients | ALS form | Disease duration (mo) | 2 ^d TP | Comparison ALS to Ctrl | Associations | References | |
|--------------|--------------|-----------------|---------------------|-----------------------|-------------------|------------------------|--------------|--------------------------------------|-----------------------------|
| Chemokines | Plasma | 29 | 18 sALS no H63D mut | 19 (12-28) | | | | Mitchell et al., 2010 | |
| | | | 11 sALS het H63D | 11 (9-19) | | | | | |
| | CSF | 39 | sALS | 16.6 (± 2.4) | | | | | Mitchell et al., 2009 |
| | | 2 | fALS | 16.6 (± 2.4) | | | | | Mitchell et al., 2009 |
| | CSF + serum | 77 | Undefined | 10 (7-18) | | | | | Martinez-Menno et al., 2018 |
| | | 20 | Undefined | 9 (± 4.4) | | | | | Kuhle et al., 2009 |
| | CSF | 42 | sALS | 13.0 (± 9.3) | | | | | Tateishi et al., 2010 |
| | | 105 | sALS | 30.70 (± 29.70) | | | | | Guo et al., 2017 |
| | CSF + plasma | 108 | 17 sALS 91 sALS | Undefined | every 4 mo for 2y | | | | Huang et al., 2020 |
| | | 26 | sALS | 14.5 (± 10.9) | | | | | Furukawa et al., 2015 |
| | Serum | 34 | sALS | 58.4 | n=34 | 8.22 (± 7.1) | No changes | Neg correlate with disease course | Simpson et al., 2004 |
| | | 14 | fALS | 58.4 | | | | | Simpson et al., 2004 |
| | CSF | | | | 58.4 | | No changes | No differences between serum and CSF | Simpson et al., 2004 |
| | | 29 | Undefined | 8 (2-24) | | | | | Wilms et al., 2003 |
| | CSF | 39 | sALS | 16.6 (± 2.4) | | | | | Mitchell et al., 2009 |
| 2 | | fALS | 16.6 (± 2.4) | | | | | Mitchell et al., 2009 | |
| CSF + serum | 58 | sALS | 24.67 (± 23.53) | n= 40 | | | | Yang et al., 2016 | |
| | 105 | sALS | 30.70 (± 29.70) | 48 | | | | | |
| CSF + plasma | 108 | 17 sALS 91 sALS | Undefined | every 4 mo for 2y | | | | Guo et al., 2017 | |
| | 77 | Undefined | 10 (7-18) | | | | | Huang et al., 2020 | |
| CSF | 42 | sALS | 13.0 (± 9.3) | | | | | Martinez-Menno et al., 2018 | |
| | 77 | Undefined | Undefined | | | | | Tateishi et al., 2010 | |
| CSF | 39 | sALS | 16.6 (± 2.4) | | | | | Gonzalez-Garza et al., 2018 | |
| | 2 | fALS | 16.6 (± 2.4) | | | | | Mitchell et al., 2009 | |
| CSF + serum | 105 | sALS | 30.70 (± 29.70) | | | | | Mitchell et al., 2009 | |
| | | | | | | | | Guo et al., 2017 | |

Table 4 Circulating chemokines and tumor necrosis factors in ALS patients' biofluids. Disease duration is expressed in mean/SEM or SD; mean/ range ; PR/ SEM in months. Tp: Timepoint

| Name | Biofluids | No of patients | ALS form | Disease duration (mo) | 2 ^d TP | Comparison ALS to | | Associations | References |
|-----------------------|---------------------|----------------|-----------------------------|---|-------------------|-------------------|------------------------------------|--|------------------------------|
| | | | | | | Ctrls | | | |
| Chemokines | IP-10/ CXCL10 | 42 | sALS | 13.0 (± 9.3) | | | | Pos correlated with DD Neg correlated with DPR | Tateishi et al., 2010 |
| | | 26 | sALS | 14.5 (± 10.9) | | | ↗ in ALS compared to MMIN | | Furukawa et al., 2015 |
| | RANTES/ CCL5 | 42 | sALS | 13.0 (± 9.3) | | | | | Tateishi et al., 2010 |
| | | 42 | sALS | 13.0 (± 9.3) | | | | | Tateishi et al., 2010 |
| | Eotaxin-1/ CCL11 | 26 | sALS | 14.5 (± 10.9) | | | ↗ in ALS compared to OND | ↗ was correlated with slower disease progression | Furukawa et al., 2015 |
| | | 51 | Undefined | 25.0 (± 2.14) | | | | | Poloni et al., 2000 |
| Tumor Necrosis Family | Plasma | 95 | Undefined | 22.4 (12.5–32.2) | | | | Patients with ALS at earlier disease stage lower TNF-α | Lu et al., 2016 |
| | CSF | 42 | sALS | 13.0 (± 9.3) | | | | | Tateishi et al., 2010 |
| | CSF | 26 | sALS | 14.5 (± 10.9) | | | ↗ in ALS compared to MMIN | | Furukawa et al., 2015 |
| | CSF | 29 | Undefined | 20 (4–125) | | | | | Martinez-Merino et al., 2018 |
| | CSF + serum | 20 | sALS hypoxemic / normoxemic | B (#11): 19.6 (±7.6) S (#9): 17.6 (± 6.92) | | | ↗ in hypoxemic ALS patients' serum | | Moreau et al., 2005 |
| | CSF + plasma | 164 | 43 FALS 121 sALS | 39.8 (9.4–227) | | | ↗ only C9 comp to other | increased level in CSF and plasma correlate with short disease | Olesen et al., 2020 |
| | CSF + plasma | 108 | 17 sALS 91 sALS | Undefined | every 4 mo for 2y | | | ↗ C9 patients | Huang et al., 2020 |
| | sTNF-RI | Serum | 51 | Undefined | 25.0 (± 2.15) | | | | Poloni et al., 2000 |
| | sTNF-RII | Serum | 51 | Undefined | 25.0 (± 2.16) | | | | Poloni et al., 2000 |

- ❖ Dysregulated TNF production has also been suggested in ALS disease. TNF- α is part of the TNF superfamily and acts as either a pro-inflammatory mediator via TNF- receptor 1 (TNFR1) or anti-inflammatory mediator via TNFR2. Most studies found increased TNF- α levels compared to controls in CSF and blood from ALS patients (Tateishi et al. 2010; Ngo et al. 2015; Lu et al. 2016; Hu et al. 2017; Blasco et al. 2017a) . While one study did not find this increase (Vlam et al. 2015). TNF- α plays a role in induction of cytokine secretions, activation or expression of adhesion molecules as well as growth stimulation. Besides these functions TNF- α has been shown to be implicated in many other cellular functions such as lipid metabolism, coagulation, insulin resistance, endothelial function (M. D. Turner et al. 2014). Thus, TNF- α pleiotropic actions make it difficult to understand its role in ALS disease.

- ❖ From the IFN family, only Interferon-gamma (IFN- γ) has been reported to be dysregulated in ALS patients compared to controls. IFN- γ is a major activator of macrophages and an inducer of Major histocompatibility class II molecule (MHC-II) expression. Increased IFN- γ levels in the CSF and blood have been correlated with faster progression and shorter survival (Tateishi et al. 2010; Juan Liu, Gao, and Zang 2015; J. Guo et al. 2017); while few studies reported a decreased IFN- γ levels in ALS patients' plasma and CSF (R. M. Mitchell et al. 2009; Lu et al. 2016).

- ❖ Colony stimulating factors are secreted glycoproteins, which act through membrane receptors and lead to intracellular signalling cascades playing a role in cell differentiation, maturation and activation. Granulocyte- and Granulocytes macrophages - stimulating factors (G-CSF; GM-CSF) have both been found to increase in the CSF and blood of ALS patients compared to controls (R. M. Mitchell et al. 2009; Ryan M. Mitchell et al. 2010; Tateishi et al. 2010; Furukawa et al. 2015; J. Guo et al. 2017).

Table 5 Circulating Interferon and colony stimulating factors in ALS patients' biofluids. Disease duration is expressed in mean/SEM or SD; mean/ range ; PR/ SEM in months. Tp: Timepoint

| Name | Biofluids | No of patients | ALS form | Disease duration (mo) | 2 ^d TP | Comparison ALS to Ctrlis | Associations | References | |
|-------------------|----------------------------|----------------|------------------|---|-------------------------|--------------------------|---|--|-----------------------|
| Interferon family | CSF | 39 | sALS | 16.6 (± 2.4) | | > | | Mitchell et al., 2009 | |
| | CSF | 2 | fALS | 16.6 (± 2.4) | | > | | Mitchell et al., 2009 | |
| | Plasma | 95 | Undefined | 22.4 (12.5-32.2) | | > | | Lu et al., 2016 | |
| | CSF | 42 | sALS | 13.0 (± 9.3) | | > | | Tateishi et al., 2010 | |
| | CSF + serum | 105 | sALS | 30.70 (± 29.70) | | > | | Guo et al., 2017 | |
| | CSF + serum | 52 | sALS | 19.17 (± 1.95) | | > | In CSF Pos correlated with DPR and month from onset In serum Pos correlated with DPR | Liu et al., 2015 | |
| | Plasma | 79 | Undefined | 21.37 (3.50-137.47) | | > | | Tortelli et al., 2020 | |
| | CSF + plasma | 164 | 43 fALS 121 sALS | 39.8 (9.4-227) | | > | > only C9 comp to other ALS or mSOD1 | Olesen et al., 2020 | |
| | Colony stimulating factors | Plasma | 29 | 18 sALS no H63D mut 11 sALS het H63D | 19 (12-28) 11 (9-19) | | > | Level associated with duration of symptoms | Mitchell et al., 2010 |
| | | CSF | 39 | sALS | 16.6 (± 2.4) | | > | | Mitchell et al., 2009 |
| CSF | | 2 | fALS | 16.6 (± 2.4) | | > | | Mitchell et al., 2009 | |
| CSF + serum | | 105 | sALS | 30.70 (± 29.70) | | > | > only in CSF | Guo et al., 2017 | |
| CSF | | 39 | sALS | 16.6 (± 2.4) | | > | | Mitchell et al., 2009 | |
| CSF | | 2 | fALS | 16.6 (± 2.4) | | > | | Mitchell et al., 2009 | |
| CSF | | 42 | sALS | 13.0 (± 9.3) | | > | | Tateishi et al., 2010 | |
| CSF + serum | | 105 | sALS | 30.70 (± 29.70) | | > | > only in CSF | Guo et al., 2017 | |
| CSF | | 26 | sALS | 14.5 (± 10.9) | | > | > in ALS compared to MMN and OND | Funukawa et al., 2015 | |

- Amongst other factors found deregulated in ALS patients' biofluids were identified, immunoglobulins (IgG), metabolic proteins, adipokines, iron-related proteins, oxidative stress (Ryan M. Mitchell et al. 2010; Ngo et al. 2015; Blasco et al. 2017a). These deregulations reflect metabolic and inflammatory dysfunctions, which may be critical actors of pathogenesis. In vitro and in vivo studies demonstrated that ALS patients IgG induced a selective motor neuron's death, presumably involving calcium (Yi et al. 2000; Obal, Siklós, and Engelhardt 2002; Pullen et al. 2004; Demestre et al. 2005). These studies support the role of immunological mechanisms in the selective loss of MNs.

All these studies have yielded different results it is thus difficult to draw a clear conclusion. An extensive meta-analysis on 71 articles including 2629 patients and 2049 controls tried to identify the main conclusions from CSF deregulated cytokines between AD, PD and ALS (X. Chen et al. 2018). In ALS, G-CSF, IL-2, IL-15, IL-17, MCP-1, MIP-1 α , TNF- α and VEGF were significantly increased in patients compared to controls. While GM-CSF, IFN-g, IL-4, IL-5, IL-6, IL-6, IL-8, IL-10, IL-12p70, IL-13, MIP1b and RANTES levels were not significantly different between ALS patients and controls (X. Chen et al. 2018). At the periphery, the inflammatory signature seemed different to that of CSF suggesting different cellular reactions. A meta-analysis regrouping 25 studies including 812 ALS patients and 639 control subjects identified a specific signature. TNF-a, TNFR-1, IL-6, IL-1b, IL-8 and VEGF were found elevated in the blood of ALS patients compared to controls. In contrast, IL-2, IL-4, IL-5, IL-10, IL-17, IFN-g, Endothelial leukocyte adhesion molecule (ELAM-1) and MCP-1 did not demonstrate significant differences between ALS patients and controls (Hu et al. 2017) . These meta-analyses appear particularly interesting as they concatenate results from different studies and help to identify consensus, nonetheless it has to be kept in mind that divergent results obtained may also be linked to the heterogeneity of ALS populations studied and should be analysed separately. Longitudinal studies would appear interesting as cytokines deregulations seem to differ between early and late phases of the disease (Ehrhart et al. 2015a; Lu et al. 2016; J. Guo et al. 2017). To date, very few longitudinal studies have been conducted, the latest one assessed patients at 6 months apart. They observed no differences between the two time points (Prado et al. 2018). Yet, the assessed population was again very heterogeneous with $3 (\pm 2.7)$ years of disease length at the baseline visit corresponding to already advanced pathology in which inflammatory events may already be stabilised (Prado et al. 2018).

Taken together, biofluids studies have strengthened previous pathological observations of the early presence of inflammation in ALS patients. However, we can hardly interpret any underlying cellular mechanism as several immune cell types often share secreted cytokines and analysis were performed at unknown stages of the pathology in ALS patients. Additionally, CSF and blood are two different compartments composed of distinct immune cells, reflecting divergent immune reactions.

Overall, neuroinflammation is a central pathological hallmark of ALS disease. However, as demonstrated by these different ways of assessing inflammatory events, markers are present yet it is difficult to know which immune cells are responsible for these responses, as well as the temporality of the events. Indeed, imaging techniques have been valuable tools to study in vivo brain gliosis without precisely identifying if microglia, astrocytes or maybe even other immune cells mediating the reaction. Additionally, biofluids examinations have identified numerous deregulated inflammatory factors in ALS patients compared to controls, again without identifying which immune cells the factors were secreted from. Although, numerous immune cell types may mediate the reaction at the same time, it would worth identifying the early driver of the inflammation in order to better orientate the therapeutic target.

3- Macrophages and microglial cells: major effectors of neuroinflammation in ALS

Myeloid cells are a large family of different cell types generally characterised as cells deriving from a myoblast lineage including granulocytes, neutrophils, basophils, eosinophils, mast cells, monocyte-dendritic cell progenitors, megakaryocytic-erythropoietin progenitors, macrophages and microglial cells. These cells are part of the innate immune system and are distributed throughout the body. They play a key role in maintaining body homeostasis and are at first line in inflammatory reactions by secreting cytokines and chemokines, migrating to sites of injury and activating the adaptive immune system by presentation of antigens. In this chapter, I will focus only on peripheral tissue resident macrophages and microglial cells in the brain. The first part will be dedicated to an overview of macrophages as innate immune peripheral effectors. In a second part, I will described microglia particularity as a unique type of tissue resident macrophages.

3.1 Macrophages peripheral effectors

3.1.1 Heterogeneity of peripheral macrophages: from monocytes derived to tissue resident macrophages

Macrophages are the main peripheral effectors of inflammation. Tissue resident macrophages are widely distributed throughout the different tissues and fulfil tissue specific and niche specific functions. Traditionally they are defined and categorised depending on their anatomical territory such as osteoclast in the bones, Kupffer cells in the liver, splenic red pulp macrophages in the spleen or lung alveolar macrophages in the lungs (Gall 1958; Davies et al. 2013a). Depending on the organ, macrophage's functions can differ, consistent with their diverse gene expression profiles amongst the different populations (Gautier et al. 2012; Davies et al. 2013b). Their identity as well as heterogeneity is established early during embryogenesis and rely on a diversity of transcriptional networks with core transcriptional programs (Mass et al. 2016).

The specification of the different macrophages subtypes are still poorly documented in humans however in rodent studies, researchers demonstrated that macrophages populations' subtypes are driven by the expression of defined repertoires of enhancers that integrate signals from the environment and elicit specific genetic programs (Lavin et al. 2014a; Gosselin et al. 2014; Bulger

and Palis 2015). Enhancers are DNA sequences, which can be bound by proteins in order to increase a specific gene likelihood to be transcribed. The proteins, which bind enhancers, are usually transcription factors. Gosselin and coworkers compared microglia to peritoneal macrophages; interestingly they found that only 4% of promoters differed with respect to histone modification signatures, while 40% of active enhancers were specific to one cell type (Gosselin et al. 2014). In the study conducted by Lavin and collaborators, seven different macrophage populations were compared and interestingly few of specific macrophage enhancers were shared among the different populations. These results revealed that activation of enhancers and epigenomic changes are influenced by tissue specific environments within which they evolve (Lavin et al. 2014a).

The importance of microenvironment on shaping macrophage phenotypes was demonstrated by directly transplanting adult mouse bone marrow into irradiated mice, which had most of their tissue resident macrophages depleted. Subsequently authors isolated macrophage populations after repletion from lung, spleen, liver and peritoneum, and found that enhancers' populations were similar to populations, which normally resided in these environments (Lavin et al. 2014). Moreover, this same study also demonstrated that transplantation of peritoneal macrophages into lungs led to upregulation of lung macrophage specific gene in these engrafted peritoneal macrophages. Although engrafted macrophages retained part of their tissue specific markers, RNA sequencing revealed 70% of differential gene expression while switching to lung macrophage phenotype (Lavin et al. 2014). The importance of the environment was also shown by several studies in which depletion of niche specific macrophages led to their replacement by circulating monocytes, which adopt the transcriptomic profile of their depleted counterpart and become long-lived self-renewing cells (Scott et al. 2016; Beattie et al. 2016). Overall, these studies demonstrate the plasticity of tissue resident macrophages and shed light on the importance of the microenvironment in shaping functional identity.

In conclusion, different macrophage population exist and although core macrophage signature genes are shared amongst the different populations, specific gene expressions are unique to each microenvironment in which they reside. Owing to their plastic nature, tissue resident

macrophages can upregulate or downregulate signature genes depending on their environment, which are mostly facilitated by their enhancers' populations and epigenetic landscapes.

3.1.2 Main functions of macrophages

Macrophages' high plasticity is owed to their inherent immune functions necessitating constant integration of external cues in order to adapt their response and maintain proper tissue homeostasis. As part of the innate immune cells, macrophages are able to respond to injury by activating and signalling their surrounding using secretion of specific factors, which is often accompanied by remodelling of external matrix and clearance of cellular debris or external pathogens via phagocytic capacities. Macrophages are also able to present antigens, albeit at a lower level compared to dendritic cells.

First line effectors of innate immunity

Within the scope of phagocytes, macrophages express a variety of receptors, which help to recognise either external pathogens or intrinsic threats linked to cellular damage. Integration of these micro-environmental cues from macrophages lead them to activate into specific phenotypes. Macrophage activation phenotypes was long thought to be a linear spectrum modelled on T helper cells nomenclature with M1 (classically activated) to M2 (alternatively activated) states of activation (Mills et al. 2000). According to this nomenclature, M1 was thought to be more pro-inflammatory while M2 phenotype had anti-inflammatory functions.

Taking the initial model of M1 and M2 response: M1 macrophages are differentiated with GM-CSF and corresponds as a "classical" activation (Fleetwood et al. 2007). M1 phenotype is evoked *in vitro* by treating cells with IFN (Toll Like Receptor (TLR) agonist) and lipopolysaccharide (LPS; bacterial cell wall component), leading to pro-inflammatory cytokines secretion such as TNF and IL-1 (Fleetwood et al. 2007; Orecchioni et al. 2019). Additionally M1 macrophages undergo metabolic transition towards glycolysis and secrete free radicals. M2 phenotype on the other hand is known as alternative activation and has fundamental roles in tissue homeostasis during development, maintenance of homeostasis and resolution of inflammation (Gordon 2003; Sica and Mantovani 2012). M2 macrophages differentiation is mediated by presence of M-CSF (Fleetwood et al. 2007). *In vitro* activation is elicited by IL-4/IL-13 and leads to increased expression of IL-10, Arginine-1 (Arg-1), Chitinase-like 3 protein (Ym1) (Wills-Karp and Finkelman 2008; Sica and Mantovani 2012; D. Zhou et al. 2014). The M2 response is now classified as the M2a response

after another form of alternative activation was identified. M1 and M2 phenotypes have distinct transcriptomic and secretion profiles. These stereotypical responses are mainly recognised *in vitro*, yet M1 and M2 type phenotypes are identifiable in physiological contexts but are less binary. In fact, markers associated with both M1 and M2 phenotypes have been observed simultaneously on individual cells *in vivo* providing evidence of macrophages phenotypic continuum *in vivo* and reveal the simplistic aspect of this polarisation paradigm (Hoeksema, Stöger, and de Winther 2012; T. D. Smith et al. 2016). The limitation of this model was pinpointed by several studies suggesting *in vivo* macrophages activation to be a more dynamic process, displaying broader transcriptional repertoire depending on environmental signals integrated (Boorsma, Draijer, and Melgert 2013; Xue et al. 2014; Guilliams and van de Laar 2015; D. Zhou et al. 2017).

Herein I will focus on two major families of patterns which are recognised by macrophages and which trigger their activation and immune response involving major macrophages functions.

- Pathogen associated molecular-patterns (PAMPs) provide exogenous signals that alert the system of the presence of external pathogens; they are present in many microorganisms but absent in the host. These patterns are essential for proper innate reaction as they help to distinguish 'self' from 'non-self' in order to adapt the reaction. PAMPs are usually microbial nucleic acids (e.g. DNS, ds-RNA, ss-RNA), bacterial peptides (e.g. flagellin), lipoproteins, surface glycoproteins or membrane components (e.g. LPS). They are recognised by the large family of Pattern recognition receptors (PRR) present on phagocytes which are sub classified according to their ligand specificity, function, localisation and or evolutionary relationships. Based on their localisation, PRR can be divided between membrane bound receptors such as Toll like receptors (TLR) and C-type lectin receptors (CLR), or cytoplasmic receptors such as NOD-like receptors (NLR) and RIF-I-like receptors (RLR). PRR signalling are complexes and will not be reviewed in details herein (X. Zhang and Mosser 2008; Kawai and Akira 2010; Tang et al. 2012). Following PAMPs recognition, PRR receptors will signal the cells the presence of microbial infection and trigger pro-inflammatory and antimicrobial downstream cascade of activation. Within the cascade, kinases, adaptor molecules (e.g. MyD88, MAL, TRIF, TRAM in TLR mammalians), transcription factors (e.g. NF-kB, AP-I, IRF) are mobilised to trigger a proper immune reaction.

- Damage associated molecular-patterns (DAMPs) are the second type of activator of innate immunity and are found during sterile inflammation, autoimmunity or injury. They are cell-derived molecules, which signal endogenous danger to trigger downstream innate immune reactions. In normal physiological conditions DAMPs are intracellularly sequestered molecules and hidden from recognition by the immune system (Land 2015). Under cellular stress, injury or other disrupter of homeostatic conditions, DAMPs can be either secreted, exposed on stressed cells, passively released in the extracellular environment from dying cells or the damaged extracellular matrix (Land 2012; Wenceslau et al. 2014). DAMPs are generally localised within the nucleus and cytoplasm (e.g. high mobility group box HMGB1), cytoplasm alone (e.g. S100 proteins, ATP), exosomes (e.g. Heat shock proteins), extracellular matrix (e.g. hyaluronic acid), and in plasma components (e.g. complement C3a, C4a, C5a) (Tang et al. 2012; Di Virgilio, Sarti, and Coutinho-Silva 2020). According to Land and collaborators, DAMPs are subdivided into five partially overlapping classes as presented in the Table 6 (Land 2015). DAMPs signal onto a variety of PRRs alike PAMPs, promoting an inflammatory response. Upon signalling on DAMPs receptors, activation of different cellular machineries ensue in order to resolve inflammation (e.g. inflammasome, unfolded protein response (UPR)).

Table 6 Classification of selected injury induced DAMPs

| Class and description | Category of respective cell bound/ humoral pattern recognition receptors/sensors |
|--|--|
| I* DAMPs recognised by binding to a receptor: including HMGB1, HSPs, fHA and nucleic acids | TLRs, RAGE, RIG-1, MDA5, cGAS, IF116 On MP, DC and other somatic cells |
| II* DAMPs sensed without direct binding to PRR: including eATP, MSU, ROS or else | NLRP3 inflammasome In MP, DC and other somatic cells |
| III* MICA, MICB, ULBPs | Activating receptors NKG2D, expressed on innate lymphocytes and innate like T lymphocytes |
| IIII* Neo antigens : including NMHC-IIA, actin cytoskeleton, oxidised phospholipids or else | Pre-existing natural IgM antibodies able to activate the complement cascade via the classical MBL and alternative pathways |
| IV* Dyshomeostasis-associated molecular patterns: Such as ER stress inducing molecules (due to accumulation of misfolded proteins, changes in acidity and osmolarity, hypoxia, oxidised proteins...) | Signalling sensors of the unfolded protein response (PERK, IRE1 α \rightarrow XBP1, ATF6) |

DAMPs = damage-associated molecular patterns; TLRs = Toll-like receptors; RAGE = receptor for advanced glycation end-products; RIG-I = retinoic acid inducible gene-1; MDA5 = melanoma differentiation-associated gene 5; cGAS = cyclic guanosine monophosphate-adenosine monophosphate synthase; IFI16 = interferon gamma-inducible protein 16; HMGB1 = high-mobility group box 1; HSPs = heat shock proteins; fHA = fragment of hyaluronan; NLRP3 = nucleotide-binding oligomerisation domain-like receptor-containing pyrin domain 3; eATP = extracellular adenosine triphosphate; MSU = monosodium urate; ROS = reactive oxygen species; MICA = major histocompatibility complex (MHC) class I chain-related protein A; MICB = MHC class I chain-related protein B; ULBPs = UL16 binding proteins; NKG2D = natural killer group 2 member D; IgM = immunoglobulin M; NMHC-IIA = non-muscle myosin heavy chain II-A; MBL = mannose-binding lectin; PERK = PKR-like ER protein kinase; IRE1 α = inositol-requiring protein 1 α ; XBP1 = spliced X-box binding protein 1; ER = endoplasmic reticulum; ATF6 = activating transcription factor 6.

PAMPs and DAMPs are a part of inflammatory mediators, which serve at activating cells in order to trigger appropriate immune reactions to resolve inflammation. PAMPs and/or DAMPs induced cellular signalling will culminate into production and secretion of a broad range of molecules such as cytokines, chemokines, adhesion molecules and else, accompanied by modification of gene expression profiles (Tang et al. 2012), presentation of antigens in order to solicit adaptive immunity and ultimately the clearance of external pathogens. These different immune linked cellular responses will be described in the next pages.

Phagocytic capacities

A specific macrophage functional property is their phagocytic capacities, which enable clearance of external pathogens and of dead or dying cells, thereby contributing to the resolution of inflammation or normal tissue homeostasis maintenance (Henson and Hume 2006; Kosmider et al. 2012; Martin et al. 2012; Arandjelovic and Ravichandran 2015; Gordon 2016).

Phagocytosis is a highly active process, and a receptor mediated event, defined by engulfment of large particles ($\geq 0,5\mu\text{m}$) within a plasma membrane-envelope termed phagosome (Gordon 2016). Phagocytosis can occur in different contexts. In the context of external pathogen infection, macrophages and other phagocytes will display specific receptors, which recognise PAMPs as were described above, foreign bodies can also be recognised by soluble molecules circulating in the blood and interstitial fluids (i.e. opsonisation mechanism); *in fine*, leading to specific phagocytic engulfment of their prey and activation of inflammatory pathway (Figure 11).

Besides external pathogen context, phagocytosis is also important for proper tissue homeostasis by effective clearance of dying cells, termed efferocytosis. In fact billions of cells are eliminated every day from healthy organisms, without triggering damage but rather being part of a homeostatic clearance program (Arandjelovic and Ravichandran 2015; Gordon 2016). This process

is governed by well-orchestrated communication between the dying cell and the phagocyte (Doran, Yurdagul, and Tabas 2020).

Different type of cell death exist linked to inflammatory or non inflammatory of cell clearance. Apoptotic cells are considered immunologically silent as they are accompanied by release of anti-inflammatory agents to prevent further tissue damage (Flannagan, Jaumouillé, and Grinstein 2012; Allen and Rückerl 2017). While necrotic cells lead to swelling of intracellular factors eliciting an inflammatory response. In the past years, other types of cell deaths have been also identified and influence the biological consequence.

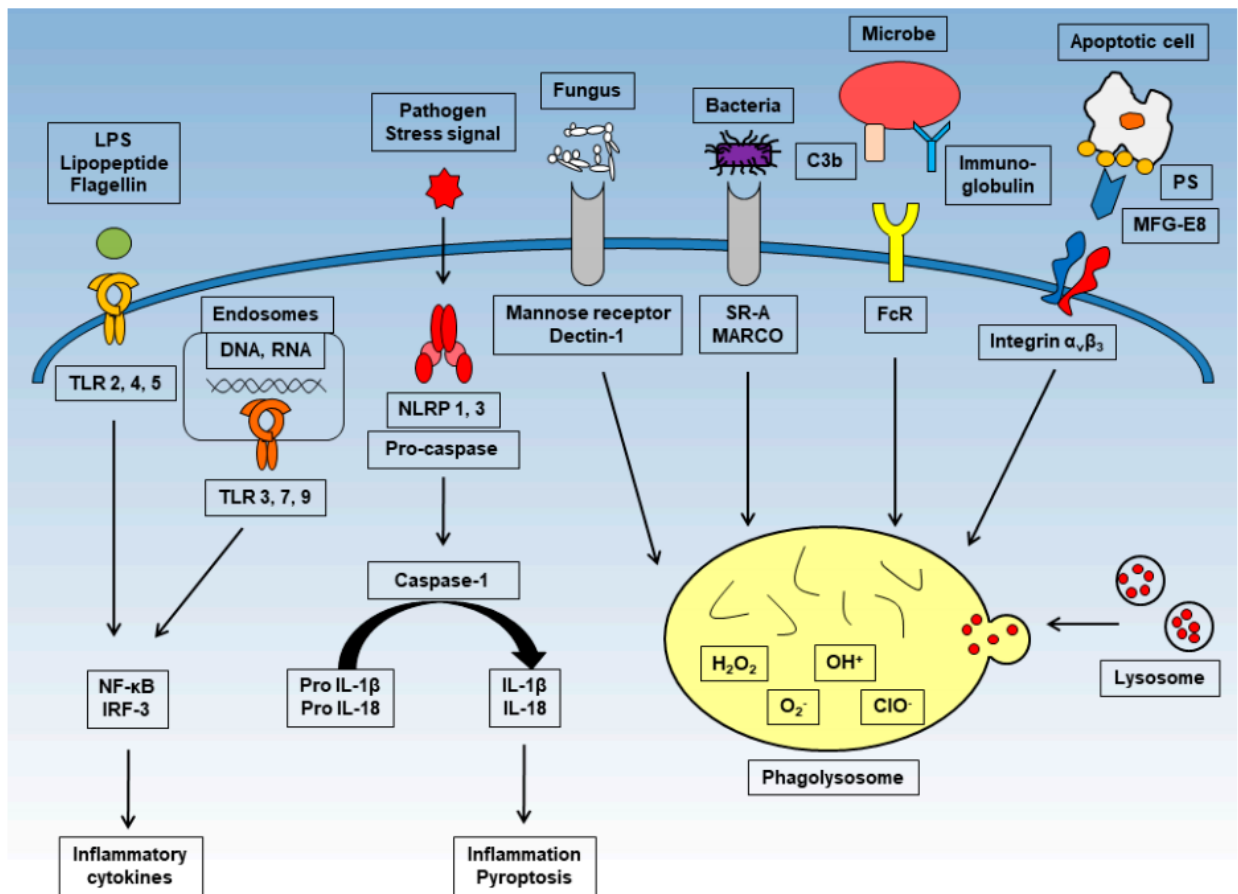


Figure 11 Inflammatory cascades elicited by phagocytosis of pathogen or apoptotic cells. Specific membrane receptors expressed in innate immune cells and which serve to recognise PAMPs and DAMPs are described on this figure. PAMPs and DAMPs can bind by direct contact with the receptor or via bridging molecules (MFG-E8) Hirayama et al., 2017

Find me signals

During apoptosis, cells release soluble signals into the milieu to attract macrophages at their vicinity. These “find me” signals are diverse including Lysophosphatidylcholine (LPC), fractalkine (CX3CL1) and sphingosine-1-phosphate (S1P), or nucleotides such as ATP, UTP (Flannagan, Jaumouillé, and Grinstein 2012; Vernon and Tang 2013; Hochreiter-Hufford and Ravichandran 2013; Doran, Yurdagul, and Tabas 2020). LPC and S1P are specific of apoptotic cell death (Boada-Romero et al. 2020). Interestingly, the “find me” signals may also serve additional roles. For instance, CX3CL1 was shown to induce Milk fat globule EGF factor 8 (MFG-E8) expression in peritoneal macrophages (Miksa et al. 2007). The latter is a bridging molecule facilitating recognition of apoptotic cells by phagocytes. Besides its role in chemoattraction, S1P was shown to provoke an anti-apoptotic and anti-inflammatory gene expression program in macrophages, characterised by suppression of TNF and IL-12 along with increased production of IL-10, VEGF and PGE2 (Johann et al. 2008; Weigert et al. 2007; Weigert, Weis, and Brüne 2009). Moreover, S1P signalling induced an anti-inflammatory phenotype in macrophages.

In contrast to apoptosis, the plasma membrane integrity is compromised in non-apoptotic cell death thereby leading to exposition of nearby cells to inflammatory signals leaking from the dying cell. Many DAMPs are released during these types of cellular death, which trigger inflammatory response and may serve as chemoattractants for macrophages (Vernon and Tang 2013).

Eat me signals

After reaching the site, phagocytes recognise “eat-me” signals on cells, which help them to distinguish dying from healthy cells. The most widely characterised “eat-me” signal, shared across all forms of cell death, is Phosphatidylserine (PS) a typical lipid of plasma membrane, which in normal conditions is for the majority located in the inner leaflet of the plasma membrane hidden from phagocytes (V. A. Fadok et al. 1992; Valerie A. Fadok et al. 1998). Exposure of PS on the surface of the cells convey the “eat –me” signal. In fact, PS binding not only functions as an activating signal, it also triggers an anti-inflammatory response mediated by secretion of IL-10 TGF β and prostaglandins while activating the pro-inflammatory cytokines secretion (Szondy et al. 2017).

A wide range of other “eat me” signals exist, such as changes in glycosylation of surface proteins, binding of serum proteins (e.g. thrombospondin), calreticulin and Intercellular adhesion molecule

3 (ICAM-3) (Kinchen and Ravichandran 2007; Ravichandran and Lorenz 2007; Kristóf et al. 2013). Phagocytes recognition can occur via direct binding of their membrane receptors (e.g. T cell immunoglobulin mucin family receptors (TIM), adhesion G protein-coupled receptor B1 (BAI1)) (Kobayashi et al. 2007; D. Park et al. 2007; S.-Y. Park et al. 2008) or via bridging elements (often soluble extracellular molecules) which help to bind phagocytes surface receptors (e.g. MFG-E8), growth arrest-specific protein 6 (Gas6), protein S) (Hanayama et al. 2002; Flanagan, Jaumouillé, and Grinstein 2012; Elliott, Koster, and Murphy 2017) (Figure 12). Moreover, PS exposed on the external surface of plasma membrane, undergoes oxidation; phagocytes can also recognise such forms of PS via CD36 and CD68 membrane receptors (Flanagan, Jaumouillé, and Grinstein 2012; Elliott, Koster, and Murphy 2017).

In parallel, healthy cells simultaneously increase their “don’t eat-me” signals, such as CD47, CD24 and the complement regulatory proteins CD200 (Vernon and Tang 2013) to prevent their mis-degradation. These signals are recognised respectively by the receptors Signal regulatory protein α (SIRP α), Sialic Acid Binding Ig Like Lectin 10 (SIGLEC-10) and CD200R respectively (Emily Kate Lehrman 2014; Barkal et al. 2019; Takimoto et al. 2019). The signal initiation to phagocyte is also mediated by restructuration of the membrane receptors. A common feature required is lateral clustering of phagocytic receptors at the site of initiation (Jaumouillé et al. 2014).

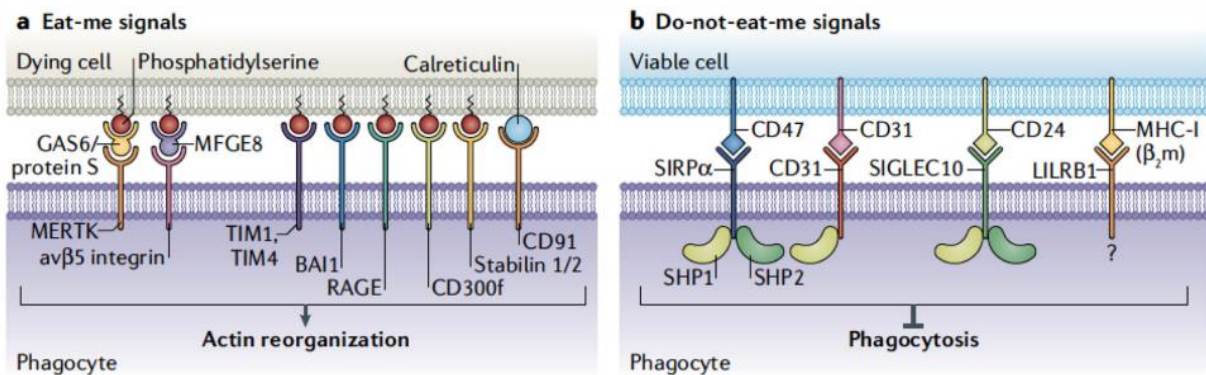


Figure 12 Cell surface signalling regulating the phagocytic process. Dying cells are exposing specific set of lipids and proteins to signal the phagocyte of their death, on the other side, healthy cells express “don’t eat me” signals to prevent mis-degradation. (a) Are presented on the right some most common “eat-me” signals on the cell surface of apoptotic cells. The most common signal is the externalisation of phosphatidylserine (PS), which can interact with a large array of receptors via direct binding of indirect binding (GAS6, MFG-E8). Calreticulin expression on the cell surface can also serve as an “eat-me” signal via binding to CD91 expressed on phagocyte. Binding of phagocyte to the dying cell elicit a remodeling of actin cytoskeleton. (b) “Do not eat me” signals are expressed on healthy cells and prevent their mis-targeting from phagocytes. These signals encompass a variety of different surface proteins. The most common include CD47 which bind to SIRP α on phagocyte, and CD31 which homodimerises with CD31 expressed on

phagocyte. Other signals are also known to convey “do not eat me” message, such as CD24 or MHC-I. Boada-Romero et al., 2020

Engulfment mechanisms and phagosome maturation

The understanding of phagosome maturation processes has been largely based on the general paradigm of receptor mediated endocytosis process. Although much of the signalling events overlap between the two processes, the phagocytic process still poses several uncertainties and remain a large area of research.

After docking onto the cell membrane, the phagocyte initiate actin remodelling. Local accumulation of activating kinases and physical exclusion of inhibitory phosphatase allow activation of receptors requiring a cytosolic domain phosphorylation. Plasma membrane invagination and extravagation leading to phagocytic cup formation and phagosome sealing are tightly regulated events involving many membrane receptors and cytosolic effectors (reviewed by Levin, Grinstein, and Canton 2016).

Once internalised the early phagosome undergoes a series of maturation events and interactions with cytosolic organelles, resulting in the remodelling of its membrane and luminal contents (Figure 13). Most of the processes underlying phagosome maturation are governed by Rab GTPase family of proteins, which alternate between active (GTP-bound) and inactive (GDP-bound) states (Harald Stenmark 2009). The process of phagosome maturation begins at the membrane with recruitment of Dynamin a GTPase to the apoptotic cell/phagocyte interface. Dynamin interacts with Vsp34, a phosphatidylinositol 3-kinase, on the phagosome leading to Rab5 recruitment at the surface. Rab5 is a GDP-bound small GTPase, and is activated by an unidentified protein (Kinchen and Ravichandran 2008). Subsequently, Rab5 activates Vsp34, leading to generation of phosphatidylinositol-3-phosphate (PI3P) at the membrane of the phagosome. PI3P is central to numerous events during early phagosome progression, actually Vsp34 inhibition arrests phagosome maturation (Fratti et al. 2001). Rab5 and PI3P tightly interact to recruit the early endosomal antigen 1 (EEA1) to the phagosome membrane (Lawe et al. 2002). Inhibition of EEA1 impairs phagosome maturation (Fratti et al. 2001). EEA1 stimulates fusion of the phagosome with the early endosome leading to early phagosome formation.

Further, the early phagosome transitions to late phagosome by switching from Rab5 positive to Rab7 positive compartment and fusion with late endocytic compartments. In the endosome pathway, this switch occurs thanks to the effector Vacuolar fusion protein (CCZ-1) which recruits Rab7 to the phagosome and drive its activation (Kinchen and Ravichandran 2010). In the phagosome pathway, the exact driver of this recruitment remains poorly described.

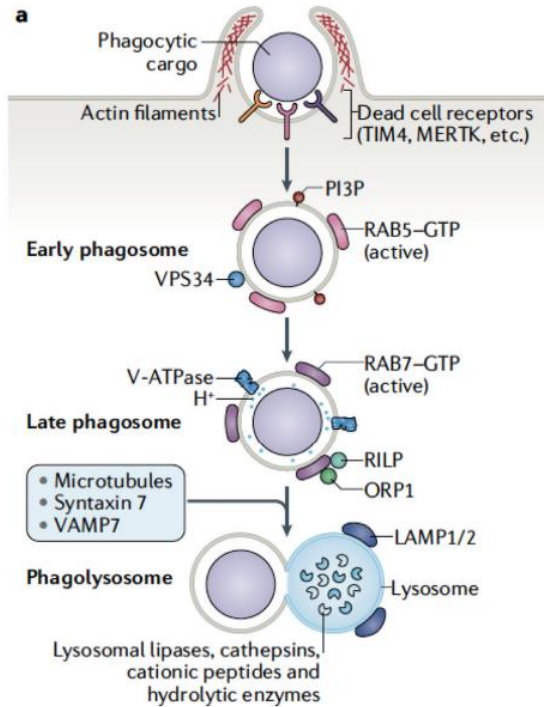


Figure 13 Schematic representation of the phagocytic process of dying cell. The degradation process involve the lysosomal pathway. (a) Schema of efferocytosis process. Early phagosome recruit Rab5 at their membrane, which allow their fusion with early endosomes. Subsequently, different molecules are recruited at the membrane allowing maturation of the early phagosome toward late phagosome. The acidification of the vesicle via V-ATPase pump culminate with their fusion with lysosomes forming phagolysosomes; Boada-Romero et al., 2020

Ultimately, after reaching a certain level of acidification, the late phagosome maturation culminates with the fusion to lysosome giving rise to a phagolysosome (Kinchen and Ravichandran 2008; Gordon 2016; Levin, Grinstein, and Canton 2016). After fusion, the latter acquires lysosomal

markers such as Lysosome-associated membrane protein-1 (LAMP1) and LAMP2 (Levin, Grinstein, and Canton 2016).

Overall, the phagocytic functions are key components of the innate immune system and is crucial for maintaining tissue homeostasis and remodelling across lifespan. Different types of sub-phagocytosis exist depending on the cargo to be cleared. Here was detailed efferocytosis which is thought to be important for clearance of cellular corpses and contributes to the maintenance of homeostasis. Additionally, many autoimmune and inflammatory diseases are associated with defects in this process with uncleared or incorrectly cleared dead cells resulting in inflammation.

Induction of secretory pathways during innate immune reaction

Upon activation, secretory functions of macrophages are one of the most important functions helping to communicate with not only neighbouring innate immune cells but also with adaptive immune cells. Macrophages harbour a portfolio of different soluble proteins able to be secreted and to communicate with neighbouring cells. They encompass: (i) cytokines to activate and recruit surrounding cells and sustain or hinder inflammation, (ii) chemokines which act as chemoattractant to the site of injury, (iii) trophic or toxic factors to support tissues, (iv) growth factors, hormones, and (v) other molecules necessary for matrix remodelling and vascular permeability. The secreted factors are of different types having distinct effects of inflammatory polarisation on surrounding cells and macrophages themselves.

Cytokines and chemokines are low molecular weight proteins conveying intercellular communications. They are secreted by a multitude of cells, primarily those of the immune system. These molecules govern a variety of processes encompassing regulation of local and systemic inflammation, cellular proliferation metabolism, chemotaxis and tissue repair. However, the main function of these factors is to regulate inflammation thus playing a vital role in regulating the immune response in health and disease.

Each cytokine acts by binding on a specific receptor generating a cell-signalling cascade that induces upregulation or downregulation of specific genes through expression of specific transcription factors. The signalling cascade can lead to production of other cytokines, increase surface receptors numbers, or even invalidate the effect of the cytokine's own effect (Arango Duque and Descoteaux 2014). Cytokines can exert their effects locally, either via autocrine or paracrine actions or can act on distant tissues through endocrine actions. A main feature of cytokine biology is their functional redundancy, allowing different cytokines to share similar functions. The secretion of cytokines is regulated by a series of inter organellar exchanges which are based in particular on vesicular traffic and cytoskeletal remodelling (Stow and Murray 2013). The secretion of cytokines is finely regulated over time. These molecules have a role in the activation and polarisation of macrophages, while also being the reflection of their state of activation. Different pro- or anti-inflammatory cytokines can be secreted by macrophages: TNF family factors, IL-1a, IL-1b, IL-1Ra, IL-6, IL-12, IL-18, IL-23, IL-27, IL-10, TGFb.

Chemokines are a family of heparin-binding cytokines able to guide cellular migration via chemotaxis (Arango Duque and Descoteaux 2014). During inflammation, chemokines are released by a wide variety of cells and involve both innate and adaptive immunity. Chemokines release is often induced by pro-inflammatory cytokines such as TNF, IL-6 and IL-1b. Amongst the most secreted chemokines by macrophages are: MIP-1 α , IL-8, RANTES, CXCL9, CXCL10, and IP-9 (Comerford and McColl 2011).

In addition to cytokines and chemokines, activated macrophages can release Reactive Oxygen Species (ROS), Reactive Nitrogen Species (RNS) and produce and secrete Inducible Nitric Oxide synthase (iNOS) (Arango Duque and Descoteaux 2014).

The different modes of macrophage activations detailed above, lead to different panels of proteins released in order to recruit potent cells to resolve the inflammation.

Activation of adaptive immunity

Alike dendritic cells which are considered the major Antigen Presenting Cells (APC), macrophages also display the capability to present antigens (Unanue et al. 1976). APC are characterised by a high expression of MHC-II or Human Leukocyte Antigen (HLA) and co-stimulatory molecules (e.g. CD80, CD86, CD40) which help to activate or counteract the activation signal received by MHC/HLA presentation. APCs are a bridge between innate and adaptive immunity. Indeed their capacity to detect, engulf and present self or foreign antigen are essential functions for targeted activation of adaptive immunity. Antigen presentation can occur in primary lymphoid organs, secondary lymphoid organs or in the tissues, herein we will be only presenting macrophages antigen presentation in tissues.

In an inflammatory context, tissue resident macrophages receive external signals as described above, which activate downstream machinery and notably upregulation of signature immunostimulatory genes (e.g. MHC-II/HLA-DR). Macrophages engulf the prey and process it in order to present a peptide fragment of it onto MHC/HLA molecules at the surface of their plasma membrane. CD4+ T effectors (CD4+ Teff) present in the tissue recognise MHC-II/HLA-DR and react to cytokines secreted by activated macrophages, which lead them to differentiate into a specific subpopulations of CD4+ Teff and thus elicit an appropriate adaptive immune response. CD4+ Teff

cell differentiation is partially dependent on cytokine combinations present in their vicinity, as described in the Table 5. After CD4+ Teff specification, they start secreting specific sets of cytokines which will sustain macrophage activation, activate differentiation of other CD4+T cells populations, recruit cells at the site of injury and may also have a systemic effect echoing onto many other cells.

Table 7 CD4 T helper cell subsets, their molecular signatures and their main effector function in immunity.

| CD4 T subsets | In vitro Differentiation cytokines | Master transcription factors | Secreted cytokines | Effector functions in adaptive immunity |
|---------------|--|--------------------------------|--|---|
| Th1 | IL-12 | T-bet STAT4 STAT1 | IFN- γ IL-2 TNF- α TNF- β | Clearance of intracellular pathogens (e.g. viruses) by cell mediated immunity such as CD8 T cell and macrophage activation (Mosmann et al., 1989) |
| Th2 | IL-4 | GATA-3 STAT6 | IL-4 IL-5 IL-9 IL-13 IL-31 | Clearance of extracellular pathogens and activation of antibody-producing B cells (humoral immunity) (Zhu et al., 2006) |
| Th17 | IL-1 β IL-6 IL-23 TGF β | ROR γ t ROR α | IL-17A IL-17F IL-21 IL-22 | Clearance of extracellular pathogens (mainly at mucosal and epithelial surfaces) Antimicrobial properties against (Liang et al., 2006) Recruitment of neutrophils (Laan et al., 1999) |
| Treg | IL-2 TGF β | FoxP3 | IL-10 TGF- β | Immune tolerance Anti-inflammatory function (von Boehmer et al., 2005) |

In conclusion, tissue resident macrophages constitute a heterogeneous population. Each subpopulation is modelled through inherent transcriptional programs as well as the integration of the environmental cues and niche specific signatures. In addition, macrophages are very potent cells endowed with many essential functions for the maintenance of cellular homeostasis. Their main functions place them at first line effectors during inflammatory events, as they are also the main regulators of these events, their dysregulation are therefore greatly associated with ND.

3.2 Microglial cells: dual belonging to glial and immune cells families

Microglial cells are the primary innate immune cells in the CNS. Population based gene expression studies have identified very distinct transcriptomic profiles in microglial cells compared to macrophages residing in other tissues, as well as amongst microglial cells between different brain regions (Gautier et al. 2012; Chiu et al. 2013; Hickman et al. 2013; Gosselin et al. 2014; Lavin et al. 2014b). In recent years, the growing interest in microglial cells has allowed a better characterisation of their homeostatic molecular signatures as well as of microglial signatures associated with diseases. These advances not only open up new avenues for understanding the normal functioning of the CNS, but also give us a new perspective on their role in ND. Ultimately, the increased understanding of microglial cells in disease, offers new ideas for therapeutic targets by specifically modulating these cells to act on a neuronal pathology.

In this chapter I will first discuss about the factors contributing to microglia activation especially in the specific environment in which they reside. Secondly I will detail the main functions of microglia cells, they of course share most of the function presented for macrophages however they still bear specific functions linked to their particular location.

3.2.1 Microglial activation regulation

Alike peripheral macrophages in the PNS, microglia act as the first line of defence in the CNS. The microglial population is not equally distributed across brain regions and may also display specific signatures depending in the region they reside (Grabert et al. 2016). Microglial ramified morphology allows them to proceed via their highly motile processes to the constant screening of the brain parenchyma (Nimmerjahn, Kirchhoff, and Helmchen 2005). These screening capacities are thought to be affected with aging leading to decreased motility of microglial processes and increased mobility of microglial soma (Hefendehl et al. 2014).

To protect the nervous system from potential harmful immune reactions, the brain provides a restricted and immunosuppressed environment termed “immune-privilege” (Galea, Bechmann, and Perry 2007). Contrary to what one might imagine, this does not mean that the immune reactions are less present, but rather that it is finely regulated (Kettenmann et al. 2011). In the healthy brain microglial activation is restricted and quickly resolved in order to avoid the spread of damage to other adjacent regions. Microglial immune functions are controlled by two classes

of signals which are “off” signals which are constitutively found in the healthy microenvironment in the CNS. Thus, loss of these “off” signals can lead to microglial responses and activation (Linnartz and Neumann 2013). On the other hand, “on” signals are more similar to those found in peripheral macrophages, and are produced on demand to initiate a defined microglial activation either pro- or anti-inflammatory. Therefore, the two signals are not mutually exclusive, although they have different modes of action.

Off signalling in microglial cells

Microglial homeostasis is kept by several factors such as TGF β in the extracellular space with immunosuppressive properties (Butovsky et al. 2014; Spittau, Dokalis, and Prinz 2020), but cell-to-cell interactions are also thought to govern most of the constitutive inhibitory signals on microglial cells. Here are presented only some important inhibitory factors of the homeostatic state of the microglial cells.

Immunoglobulin super family

Members of the immunoglobulin super family (IgSF) are often downregulators of myeloid cell function. The best-characterised molecules belonging to the IgSF in the CNS are CD200 and CD47. Both of these markers are constitutively expressed on the neuronal membrane surface, oligodendrocytes and astrocytes (Wright et al. 2001; Barclay et al. 2002). While the CD200R is exclusively present on microglia and other CNS macrophages. The contact between CD200-CD200R confers a negative signalling, providing constitutive inhibitory signals to microglia (Biber et al. 2007). Loss of this inhibitory signal leads to microglial activation, change of morphology and upregulation of specific markers. In parallel, CD47 is a regulatory signal, which is mostly found at the periphery as a “do not eat me” signal (Jaiswal et al. 2009). CD47 is expressed on neuronal cells and it was shown that during development the expression of CD47 is located at the synaptosomes, which are not targeted for degradation. On the other side, microglial cells express CD172a/SIRP α the receptor recognising CD47 and thus permit these synaptosomes to be protected from degradation (Figure 12) (Biber et al. 2007; Emily K. Lehrman et al. 2018). This signalling is also thought to be important in the tumour environment, which uses it as a way of escaping degradation (Hutter et al. 2019).

CX3CR1 inhibitor of microglia neurotoxicity

The G protein coupled seven transmembrane C-X-3C chemokine receptor 1 (CX3CR1) is expressed in diverse immune cell types including monocytes, macrophages DC, NK, but in the CNS the expression is restricted to microglial cells (Wolf et al. 2013). Its only known ligand, CX3CL1 or fractalkine, is constitutively expressed on different neuronal subtypes (K.-W. Kim et al. 2011) and can be either secreted or at the membrane (Hughes et al. 2002). Nevertheless, it is still not known if the membrane or soluble CX3CL1 is responsible for microglia inhibition in vivo. An important role of CX3CL1-CX3CR1 signalling in neuron and microglial interaction has been reported (Biber et al. 2006; Cardona et al. 2006). Under physiological conditions, it is thought that synaptic pruning may be governed by CX3CR1-CX3CL1 signalling, as CX3CL1 is highly expressed during synapse maturation (Paolicelli et al. 2011; Hoshiko et al. 2012). Loss in CX3CR1 was reported to lead to decrease neurogenesis, as well as cognitive and synaptic plasticity impairments (Rogers et al. 2011; Bachstetter et al. 2011). Although these results are still controversial in different neurodegenerative conditions, it is suggested that CX3CR1 could play divergent roles under inflammatory or degenerative conditions (Prinz and Priller 2010; Prinz et al. 2011; M. Lee et al. 2018). Taken together, this demonstrates the pleiotropic action of CX3CR1-CX3CL1 signalling and the importance of this signalling cascade for proper brain development and homeostasis.

TREM2 –DAP12 axis

Another important microglial regulator is the Triggering Receptor Expressed on Myeloid cells 2 (TREM2). It associates with the adaptor protein DNAX-activating Protein of 12-kDa (DAP12), leading to activation of downstream signalling cascades. TREM2 and DAP12 interaction is thought to mediate a number of inflammatory responses linked to phagocytosis, modulation of pro-inflammatory cytokines production and transcriptional changes leading to microglial proliferation and survival (H. Neumann and Takahashi 2007; Jay, von Saucken, and Landreth 2017; Konishi and Kiyama 2018). However, it remains unclear if TREM2 is a pro- or anti-inflammatory molecule, as depending on the context it convey different microglial activations (Konishi and Kiyama 2018). In the past years, TREM2 was identified as an important risk factor for several ND, including AD, FTD and ALS (Guerreiro et al. 2013; Jonsson et al. 2013; Cady et al. 2014; Konishi and Kiyama 2018; S.-L. Zhou et al. 2019). Moreover, a recent study identified in a mouse model of AD a type of microglia, which they named “disease associated microglia” (DAM). The latter is suggested to be

associated with ND and have decreased homeostatic gene signature and increased activation genes such as Apolipoprotein E, TREM2, Lipoprotein Lipase and more (Keren-Shaul et al. 2017). The authors suggested that the initial microglial activation was Trem2-independent, and that Trem2 was only contributing in a second time. Another study also identified DAM and suggested that TREM2-APOE pathway to be responsible for the switch from homeostatic microglia towards neurodegenerative microglia (Krasemann et al. 2017). Modulation of this axis permitted the restoration of the homeostatic signature of microglia in ALS and AD mouse models (Keren-Shaul et al. 2017; Krasemann et al. 2017). Interestingly, the TREM2/DAP12 axis seems to be central in the activation process of microglia. A better understanding of the function of TREM2/DAP12 in disease context may offer new therapeutic strategies for ND.

In addition to these specific membrane regulators, several studies showed that age and CNS regions might display different homeostatic microglia phenotypes (Olah et al. 2011; Holtman et al. 2015; Galatro et al. 2017). The complexity of the microenvironment composed of many different cells with different activation states all having an important role in the maintenance of homeostasis, suggest that the “off” regulation involves certainly a number of other receptors which may be implicated in various other cellular functions (Linnartz and Neumann 2013).

The on signals

In contrast to “off” signals which are present during homeostasis, “on” signals are present in endangered or impaired neurons and can initiate protective or detrimental microglia functions. Following detection of injury signs, often directed by release of ATP from neighbouring cells, associated with extracellular calcium waves, microglial cells rapidly move towards the lesion (Davalos et al. 2005; Sieger et al. 2012). Microglia thus switch from a homeostatic state to an reactive state, which encompass a large range of activations depending on the particular signal detected (DAMPs, PAMPs). Similarly to the polarisation capacities described in macrophages above, microglial cells also display a large panel of activations and associated receptors. I will present several microglial activators.

Purines as signalling factor

Damaged or overactive neurons release or leak purines, including ATP and UTP to signal neighbouring cells of their affliction (Davalos et al. 2005; Sieger et al. 2012; Di Virgilio, Sarti, and

Coutinho-Silva 2020). ATP acts as a danger signal in the extracellular space where it is short lived, and is thus considered as one of the main DAMP molecules (Eltzschig, Sitkovsky, and Robson 2012; Vénéreau, Ceriotti, and Bianchi 2015). Microglial express numerous purines receptors at their membranes including P2X4, P2X7, P2Y2, P2Y6 and P2Y12 (Calovi, Mut-Arbona, and Sperlágh 2019). Purinergic receptor family are categorised in two different subfamilies. P1 are metabotropic receptors sensitive to adenosine. On the other hand, P2 receptors are activated by nucleoside di and tri phosphates and are subclassified into P2X and P2Y receptors. P2RX are ionotropic receptors, which are ATP gated ion channels permeable to cations (Vénéreau, Ceriotti, and Bianchi 2015; von Kügelgen 2019). P2RY are metabotropic receptors, which are G-protein coupled, and are activated by purines and pyrimidines.

P2 metabotropic signalling has been linked with chronic inflammation (Idzko, Ferrari, and Eltzschig 2014). The most abundant ligand for P2YR is ATP. In the brain, P2Y purinoceptor 12 (P2RY12) is highly expressed on ramified processes of microglia and is one of the main gene of the core microglial signature. The first step of microglial activation, is the stimulation of P2RY12 leading to their protrusion extension towards the site of injury (Rivera, Vanzulli, and Butt 2016). The migration towards the source of ATP release relies on the interaction between P2RY12 and P2RX4. Subsequently, microglia adopt activated amoeboid morphology and depending on the purinergic receptor involved will elicit phagocytosis (P2RY6), pinocytosis (P2RY4) and secretory activity (P2RX4 and P2RX7) (Koizumi et al. 2013; Gómez Morillas, Besson, and Lerouet 2021). Microglia chemotaxis via P2RY12 signalling was shown to be essential to clear damaged cells, cellular debris, tissue repair as well as to maintain intact BBB (Lou et al. 2016; Smolders et al. 2019).

Chemokines

Damaged or suffering neurons in the CNS release chemokines, which will attract immune cells at its vicinity in order to resolve the threat. These chemokines can have also direct effects on neuronal cells and mediate cross-talk between neurons and microglia (Cartier et al. 2005). Chemokines can have protective role as I have described earlier with CX3CL1-CX3CR1 binding which reduces TNF α and NO in activated microglia (Sokolowski et al. 2014). However, they can also have toxic role for instance by recruiting and activating microglia and cause neuronal death especially in disease conditions (Cartier et al. 2005; Sui et al. 2006). Besides their role in

inflammation chemokines are shown to be involved in neurodevelopment, modulation of synaptic transmission as well as cell proliferation and trophic support (Rostène et al. 2011).

Glutamate

Excess presence of neuronal glutamate in the extracellular space is associated with neurodegenerative processes and thus triggers activation of microglial cells (Pocock and Kettenmann 2007). In vivo and in vitro primary mice or rat microglia culture express a diversity of glutamate receptors (GluRs) (e.g. AMPA-type, GluR1-4, kainate receptors, mGluR) (Pocock and Kettenmann 2007; Kettenmann et al. 2011). The different groups of metabotropic glutamate receptors (mGluR) have diverse roles on microglia. The activation mGluR group II receptors in vitro induced important release of TNF- α and resulted in neurotoxic microglial phenotype that could be attenuated by specific antagonist (D. L. Taylor et al. 2002). On the other group III, mGluR activation in vitro activated mildly microglia but not in a neurotoxic way and reduced LPS neurotoxicity. Thus suggesting that group III mGlu receptors modulated the production of microglial-derived neurotoxins (Deanna L. Taylor, Diemel, and Pocock 2003). Thus, this demonstrates the fine regulation of microglial activation, which exists with the constant integration of the various environmental factors contributing to a more or less neurotoxic activation according to the needs.

Sexual dimorphism

During brain developmental stages it has been shown that gender has a marked importance in microglial morphology and cellular function in several brain regions in rodents (Schwarz, Sholar, and Bilbo 2012; Nelson, Warden, and Lenz 2017). This sexual dimorphism was shown to persist in the adult brain, and in the recent years, particular interest has been given to microglial cells in sexual dimorphism in healthy and diseased brains (Villapol, Loane, and Burns 2017; Villa, Della Torre, and Maggi 2019). In fact, growing evidences suggest that gender may have prominent role in immune cell polarisation, activation and subsequent inflammatory responses. Knowing that gender is a major risk factor in several ND, several groups have focussed their interest in the role of sexual dimorphism in microglial reactions in ND. Interestingly, in AD, changes in genes linked to microglial activation were shown to be different between males and females in APP/PS1 mice (Guillot-Sestier et al. 2021). Additionally, marked microglia morphological and activation differences between male and female were reported in APP/PS1 mice and AD patients' post

mortem tissues (Guillot-Sestier et al. 2021). In ALS microglial cells sexual dimorphism have poorly been studied, and would warrant further attention (Hunter et al. 2021).

Taken together, “on” and “off” signalling are simplistic manners to define the mode of action, not the outcome of the activation. Looking at the diversity of receptors and microglial populations in the brain this paradigm may be oversimplified and will certainly change in the next few years with discoveries of specific subtypes of microglial populations especially in the context of diseases. Recent studies have already started to identify specific time and region dependent subtypes of microglia in mice and human brains, harbouring unique transcriptomic signatures and which may respond differentially upon brain aggression (Masuda et al. 2019; Stratoulis et al. 2019; Sankowski et al. 2019; Geirsdottir et al. 2019; H. S. Yang et al. 2021). Additionally, in the recent year other factors were shown to have prominent roles on microglial cells activation, such as sexual dimorphism. As a number of ND have gender incidence differences, it would be interesting to further study the role of sexual dimorphism in microglial cells activation in ND.

3.2.2 Main cellular functions across lifespan

Microglial cells being part of the innate immune cells they harbour most of the immune functions shared between phagocytes, such as phagocytosis, cytokines and chemokines production, pro- and anti-inflammatory responses (Czeh, Gressens, and Kaindl 2011). However, due to their particularity in residing in an immune privilege area, they are less potent APC; albeit during inflammatory processes microglia upregulate their MHC-II and thus contribute actively to antigen presentation to neighbouring CD4+ cells which have infiltrated the parenchyma (Voß et al. 2012; Mittal et al. 2019; Subbarayan et al. 2020). The APC capacities of microglia are still an active area of research. In addition to these functions which are shared between tissue resident macrophages, they also display a role in immune surveillance due to microglial particular localisation through extra and intra cellular specific receptors. Besides their immune functions, microglia harbour also a panel of neuronal functions, including synaptogenesis, and plasticity, neurotrophic support, neurogenesis. The immune functions were already detailed in the tissue resident macrophage chapter (3.1.2). I will here focus on the specific trophic support functions of microglial cells as they represent a major interest for my work.

Microglial cells are also the main source of neurotrophic factors secretion supporting neuronal wellbeing. Amongst the secreted factors are nerve growth factor (NGF), BDNF, neurotrophin 3 (NT3), fibroblast growth factor (FGF) and insulin-like growth factor 1 (IGF1) (Butovsky et al. 2014; Frost and Schafer 2016). Several studies have shown the importance of microglial trophic support during the development and postnatal life. For instance, in vitro neurons treated with conditioned media from primary rat microglia, enhanced neuronal survival by 50% compared to untreated neurons (S. C. Morgan, Taylor, and Pocock 2004). Microglial trophic support has also been shown in vivo, using CX3CR1 knockout mice in whom a significant increase in the number of apoptotic neurons was reported. This effect was thought to be attributed to reduction in IGF1 signalling rather than deficiency in inefficient clearance of dead cells. These findings were supported by another study that also reported the critical need of microglial secreted IGF1 for proper neuronal survival in cortical neurons of Layer V in mice during postnatal development (Ueno et al. 2013). Microglial cells can also bring trophic support in response to injury. For instance, following striatal injury activated microglia induced expression of GDNF factor mRNA in mice. Additionally, BDNF was expressed in and around the wound and contributed to dopaminergic neurons sprouting (Batchelor et al. 1999). However, there seems to be a duality of the effects with both protective and neurotoxic roles in a time and region dependent manner (Kigerl et al. 2009; X. Zhou, He, and Ren 2014; Gaudet and Fonken 2018).

In conclusion, microglia cells bring trophic support to their local micro-environment in the CNS by clearance of cellular debris and secretion of trophic support. However, upon injury microglia may also become more toxic in reaction to the increased inflammatory environment.

4- Implication of the immune cells in ALS pathogenesis

ALS linked genes are ubiquitously expressed and importantly they are mostly involved in critical cellular functions suggesting that disruption of cellular homeostasis with time may be one of the prominent reasons for the disease to appear. Although the underlying pathomechanisms may differ between ALS forms as the impacted genes and subsequent functions may be different, all ALS patients present protein aggregates in neuronal and glial cells of the spinal cord. Moreover,

90% of ALS patients present with TDP-43 inclusions although they do not present mutations in this gene suggesting possibly similar affected pathways between different ALS forms.

ALS disease is a non-cell autonomous disease, meaning that the pathology solely in the affected neuronal population is not sufficient to induce the disease. For many years, researches have focussed largely on MNs intrinsic defects, which are possibly responsible for their vulnerability to the disease. Nonetheless, it is now well admitted that ALS pathology implies numerous other cells. Thus, nowadays, ALS is appreciated as a multifactorial and multisystem disease. We will in the next section first discuss about the gold standard SOD1 mouse model, which paved the way in building hypotheses regarding ALS pathogenesis. Secondly, will be presented the genetic evidences of the implication of immune cells in ALS pathogenesis. Thirdly, I will discuss about the evidences of microglial and macrophages implication in ALS pathology. Lastly, will be presented the implication of other immune cells in ALS pathogenesis.

4.1. SOD1 mouse model paved the way

The SOD1 mouse model was developed following the discovery of the gene SOD1 implication in ALS disease in 1993. This model has been a gold standard and a valuable tool to examine many hypothetical pathomechanisms underlying ALS pathogenesis. Mice over-expressing various human variants of mutated SOD1 develop progressive MN pathology reminiscent of the human ALS disease (B. J. Turner and Talbot 2008). At the histopathological level, mice also exhibit human pathology with presence of SOD1 aggregates without TDP-43 inclusions. SOD1 transgenic mouse model has served not only to unravel many early pathological mechanisms in MNs but also highlight the importance of non-neuronal cells in the disease progression (Boillée et al. 2006a; Beers et al. 2006a; Yamanaka, Chun, et al. 2008; Yamanaka, Boillee, et al. 2008; Lobsiger et al. 2009). The use of cre-lox system under specific promoters allowed to show how the specific overexpression of human mutant SOD1 in different cell types could have major importance on disease onset, progression or disease survival. The expression of human mutant SOD1 in astrocytes, oligodendrocytes, macrophages and microglia cells all had detrimental role in the pathology as decreased expression of the human transgene in these cells could ameliorated the disease. In contrary, human mutant SOD1 expression in peripheral Schwann cell did not prove any toxicity

and might even be protective, as specific removal of around 70% of mutant SOD1 expression accelerated disease progression (Lobsiger et al. 2009; L. Wang et al. 2012).

Taken together, although the mutant SOD1 mouse models represent only a fraction of ALS cases, it is an integrated model that recapitulates ALS patients' pathology in many aspects. The use of the mutant SOD1 mouse model allowed a better understanding of the chronological events of disease genesis as well as the identification of many faulty mechanisms. Furthermore, it allowed a more comprehensive view of the neighbouring cells' involvement ALS pathogenesis, which broaden our insight in possible targets.

4.2 ALS genes involved in immune function linked to inflammation

In recent years, advances in whole genome sequencing techniques have led to the discovery of several new ALS-linked mutant immune genes, providing evidence that immune dysregulation can both initiate and contribute to ALS pathogenesis. Mutations in these immune related genes are considered to be rare in ALS cases, however they allow new insights in potential dysregulation which could lead to ALS phenotype. These mutant genes are optineurin (OPTN), serine-threonine protein kinase binding kinase 1 (TBK1), sequestosome-1 (SQSTM1), tumour necrosis factor alpha-induced protein 3 interacting protein 1 (TNIP1), valosin-containing protein (VCP) and chemokine receptor 1 (CX3CR1). In addition, ALS most common genes have also been shown to be involved directly or indirectly with innate immune inflammation. C9orf72 has been shown to be linked with the degradation machinery but also has a role in the immune system by regulating the trafficking and lysosomal degradation of inflammatory mediators to affect inflammatory outputs (Pang and Hu 2021). On the other hand, SOD1 and TDP-43 mutations have also been implicated directly and indirectly in inflammatory event: directly by the fact that both genes have shown to promote increased inflammatory phenotype in innate immune cells, and indirectly, by the fact that the mutated proteins interact with the cellular machinery (Deora et al. 2020) (Table 8).

Table 8 ALS mutant genes which are thought to have a link with the innate-immune inflammatory responses. Adapted from Beers et al., 2019

| Genes | Function | References |
|----------------|---|--|
| C9orf72 | Expressions enhance microglial pro inflammatory priming with impaired regulation of the autophagy and lysosomal pathway | Majounie et al. 2012; DeJesus-Hernandez et al. 2011; Renton et al. 2011; Lall and Baloh 2017; Higginbottom 2016; Haeusler et al. 2014; Sullivan et al. 2016; ORourke et al. 2016; Shao et al. 2019 |
| CX3CR1 | Mutations impair a major pathway of microglia-mediated neuroprotection | Lopez-Lopez et al. 2014; Calvo et al. 2018 |
| OPTN | Mutations activate the neuroinflammatory NF-κB pathway and impair autophagy | Akizuki et al. 2013; Markovinovic et al. 2018; Bussi et al. 2018 |
| SOD1 | Mutations enhance microglial pro-inflammatory priming | Philips and Robberecht 2011; Rosen et al. 1993; Beers et al. 2006; Boillée et al. 2006; Chiot et al. 2020; Deora et al. 2020 |
| SQSTM1 | Mutations impair autophagy and increase oxidative stress by altering the phosphorylation of OPTN and SQSTM1/p62 | Fecto et al. 2011; Mitsui et al. 2018 |
| TBK1 | Mutations impair autophagy by altering the phosphorylation of OPTN and SQSTM1/p62 | Bussi et al. 2018; Duan et al. 2021; D. Xu et al. 2018; A. S. Moore and Holzbaur 2016; Freischmidt et al. 2017; de Majo et al. 2018; Oakes, Davies, and Collins 2017 |
| TARDBP | Mutations enhance microglial pro-inflammatory priming | W. Zhao et al. 2015; Neumann et al. 2006; Correia et al. 2015; LaRocca et al. 2019; Deora et al. 2020 |
| TNIP1 | Mutations activate the NF-κB inflammatory pathway | Benyamin et al. 2017; Gateva et al. 2009 |
| VCP | Mutations impair overall protein degradation by the ubiquitination-proteosome pathway and autophagy | Abramzon et al. 2012 |

Altogether, the identification of these immune-linked ALS genes have reinforced the idea of a central role of immune cells in ALS pathogenesis and possible target for treatment. Although these genes are only representative of a small fraction of ALS cases, they offer new perspectives on mechanisms, which could be involved in most ALS cases. Indeed, inflammation being a shared pathological hallmark between ALS patients, it is necessary to understand when and how it should be modulated in order to better target the disease.

4.3 Current knowledge regarding microglia and macrophages implications in ALS pathogenesis

Non-neuronal cells contribution in ALS pathology is now well admitted. As mentioned above different types of glial cells (astrocytes, oligodendrocytes, and schwann cells) are playing critical role in disease progression and represent possible targets for therapeutic interventions. In this part, I will focus on the role of microglia and macrophages in ALS pathology.

4.3.1 Microglial component in ALS pathogenesis

Microglial activation has been observed in the motor cortex as well as in the spinal cord of ALS patients post mortem tissues (Dols-Icardo et al. 2020; Brettschneider et al. 2012; D'Erchia et al. 2017), although microgliosis levels seemed to differ between ALS forms being more present in SOD1 mutated patients compared to other ALS subtypes (Spiller et al. 2018). The question as to whether microglial cells are beneficial or toxic for MNs survival is still a debated question. Today it seems that wild-type microglia is beneficial for MNs survival by helping to clear-up cellular debris and dying MNs, however the mutations in immune cells seem to have a deleterious role on these cells and may contribute to their activation and toxicity in neuronal support (Boillée et al. 2006a; Beers et al. 2006a; J. C. Lee et al. 2012; Martínez-Muriana et al. 2016; Spiller et al. 2018).

Microglia cells directly contribute to MNs degeneration in SOD1 ALS mouse models

Early studies have shown that by downregulating mutant SOD1 G93A and G37R transgene expression specifically in myeloid cells, mice had an increased disease duration with prolonged late-phased pathology without affecting the onset of the disease (Boillée et al. 2006a; Beers et al. 2006a). This suggests a toxic effect of mutant SOD1 in microglial cells. In support of these findings, CNS replacement of mutant microglia by wild type microglia slowed disease progression and increased survival of these mice (J. C. Lee et al. 2012). More recently, a study pharmacologically inhibited CSF1R, a protein essential for microglia proliferation, survival and maturation. This inhibition of CSF1R reduced microglial activation and extended survival of mutant SOD1 G93A mice (Martínez-Muriana et al. 2016). Regarding the temporality of microglia contribution to ALS pathology, it has been suggested that microglia implication in SOD1 ALS pathology is an early event. In fact, microglia modifications preceded disease onset in SOD1 G93A, and has been also confirmed with transcriptomic studies which observed increased expression of microglial

activation genes before disease onset (Alexianu, Kozovska, and Appel 2001; Y. N. Gerber et al. 2012; Maniatis et al. 2019; Chiot et al. 2020).

Overall, to date most of the studies which showed a direct link between the expression of the mutant form of the protein in the microglial cells and the progression of the pathology were carried out in the ALS SOD1 mouse model. The temporality of the microglial contribution in other ALS subtypes remain poorly documented.

Temporality of microglial cells activation in ALS

Microglial cells have different morphologies depending if they are surveilling or activated. A comparative morphometric analysis studied the changes of microglial morphology in different stimulated environments. They showed that in the early phase of the disease in SOD1 G93A mice, microglia displayed a small cell body with short and simple processes. As disease progresses microglia develop a bushy shape with a larger cell body (Ohgomori et al. 2016). These changes in morphology reflect changes in their activation, which is thus thought to evolve along disease progression. In accordance with these observations, in the mutant SOD1 mouse model, anti-inflammatory profile of microglia is observed at the pre-onset phase with high levels of IL-10 cytokine and attenuated TLR1 response (Gravel et al. 2016). Subsequently, at disease onset and during the slow progressing phase, anti-inflammatory markers are also predominantly expressed in the lumbar spinal cord of ALS mice (Beers, Zhao, et al. 2011; Hooten et al. 2015), and microglia isolated from disease onset mutant SOD1 mice, were neuroprotective towards MNs (Liao et al. 2012; Chiot et al. 2020). These results suggest that during the early phase of the pathology microglial cells, even if bearing the mutations, had a beneficial effect towards MNs survival by creating an anti-inflammatory environment. However, towards the end-stage, in several ALS models, microglial cells switch to a more pro-inflammatory phenotype, with increased NOX2 and other inflammatory markers thought to be deleterious for neighbouring cells including remaining MNs (Beers, Zhao, et al. 2011; Liao et al. 2012; Hooten et al. 2015; Funikov et al. 2018). Nonetheless, a growing body of evidence leads to reconsider this clear binary categorisation of the neuroinflammatory response; indeed, polarisation in one state or another is strictly speaking reductive, as it is now evident that microglial activation is more complex (Ransohoff 2016; Dubbelaar et al. 2018; Yuan, Wu, and Ling 2019; Chiot, Lobsiger, and Boillée 2019). Deep RNA sequencing studies which isolated microglia cells at different disease phases reported the

expression of both anti- and pro-inflammatory genes at the same time (Suh et al. 2013). Finally, it would seem that the temporality of the inflammation during disease course is important and that intrinsic defects in immune cells and extrinsic factors can contribute to the polarisation of the immune cells. A better understanding of these factors that lead to the activation of immune cells would help to better target critical inflammatory time windows in ALS patients.

The drivers of microglial cells activation: intrinsic defects

It is somewhat difficult to dissociate in vivo intrinsic defects from extrinsic activators as both are linked. The main intrinsic defect which could contribute to cell activation is certainly the mutation itself, in cases where a genetic link has been made. However, in most cases no genetic factors have been reported in the patients and still the immune inflammation is present suggesting that other intrinsic defects may contribute to this activation. Herein will be presented several intrinsic defects which could contribute to the activation of microglial cells in ALS pathogenesis.

Nuclear Factor - κ B (NF- κ B)

Nuclear Factor - κ B (NF- κ B) is a transcription factor regulating multiple aspects of innate and adaptive immune functions. NF- κ B has a pivotal role in inflammatory responses by inducing pro-inflammatory genes and regulating inflammasome (Jia Liu and Wang 2017). Interestingly, in the SOD1-G93A mouse model, specific inhibition of NF- κ B in astrocytes did not increase motor function or survival; however, selective inhibition in microglia rescued MNs survival in vitro and in vivo and extended survival in ALS mice by significantly delaying disease progression (Crosio et al. 2011; Frakes et al. 2014). Conversely, in WT mice constitutive activation of NF- κ B specifically in myeloid cells created spinal cord pathological features similar to that of ALS patients, such as gliosis and MNs death (Frakes et al. 2014). Altogether, these data revealed the importance of NF- κ B activation in microglia, becoming neurotoxic towards MNs.

Interestingly other ALS genes are shown to have direct functions in regulating NF- κ B activation, and mutations in these genes could directly impair this cellular pathway:

- Optineurin is a negative regulator of TNF- α induced NF- κ B activation. Loss of function mutations in this gene have been reported in ALS patients and thus could contribute to microglial cells activation (Maruyama et al. 2010).
- TBK1 is a NF- κ B activating kinase recognised for its major functions in the production of pro-inflammatory cytokines.

Overall, NF- κ B dysregulation in ALS innate immune cells may be a major actor of non-cell autonomous toxicity in the pathology.

Reactive oxygen species (ROS) and reactive nitrogen species (RNS)

Glial cells are considered among the major producers of ROS and RNS in the CNS under pathological conditions (Lugrin et al. 2014). Several sources of ROS exist within the cell. Firstly there are the biological processes releasing ROS as a byproduct, and secondly there are those processes that generate ROS intentionally, either as a signal for other downstream pathways or part of a defense mechanism (M. J. Morgan and Liu 2011). In the first case, the mitochondria is the main source of ROS, since oxidative phosphorylation processes frequently lose electrons during their transfer between electron transport chain complexes, which react with molecular oxygen to generate ROS. In these cases, SOD, catalase or other enzymes are helping to prevent oxidative damage. The second source of ROS are from enzymes which generate ROS for diverse purposes. The most common enzyme generating ROS is the NADPH oxidase, NOX2 (gp91), which uses NADPH to reduce molecular oxygen to produce superoxide (D. I. Brown and Griendling 2009). This superoxide is used as a defense tool against infectious pathogens. In the phagosomal compartment, superoxide is converted to hypochlorous acid, a potent microbicidal compound (M. T. Quinn, Ammons, and DeLeo 2006). During this transformation, some leakage of superoxide anion or ROS can occur and enter the cytosol contributing to oxidative stress in the cell. Besides the role in antimicrobials, NADPH oxidases have been implicated in production of ROS by growth factors, cytokines, although they can be also generated by other means (M. J. Morgan and Liu 2011). Thus, there are many intracellular sources of ROS, much of which can influence or be influenced by NF- κ B activity. In fact, in ALS disease, mitochondrial defects have been reported and oxidative stress is one of the hypotheses for MNs stress and degeneration (H. Kawamata and Manfredi 2010; Palomo and Manfredi 2015; Gautam et al. 2019). NOX2 expression is increased both in human and mice microglial cells across disease suggesting that oxidative stress in microglia cells may be an important factor driving neuroinflammation (Apolloni et al. 2013; Seredenina et al. 2016). In SOD1 linked ALS mice, NOX2 knockouts significantly slow disease progression (Marden et al. 2007); however these findings were not confirmed by Seredenina and collaborators study (Seredenina et al. 2016). In physiological conditions, SOD1 was shown to control GTP hydrolysis by Rac1. However, ALS mutant forms of SOD1 stabilises the Nox2 activator Rac1-GTP, thus leading to

over producing ROS in ALS microglia (Harraz et al. 2008; Boillée and Cleveland 2008). Moreover, TDP-43 in MNs was shown to cause sensitivity to oxidative stress. Aggregated TDP-43 sequestered some essential mitochondrial microRNA, leading to global mitochondrial imbalance which further exacerbated oxidative stress (Zuo et al. 2021). Knowing that TDP-43 aggregates are found in most of the ALS cases, we can imagine that in active immune cells, TDP-43 aggregates in microglia may also impact mitochondrial functions and increase oxidative stress. In spite of its intracellular effect, control or mutant TDP-43 binding to CD14 on microglial cells was shown to upregulate NOX2, TNF- α and IL-1 β production, and induced neuronal toxicity (W. Zhao et al. 2015). The interplay between oxidative stress and neuroinflammatory response is a vicious circle, as pro-inflammatory factors induce formation of reactive species, and in parallel free radicals lead to signalling pathways converging in the activation of NF- κ B, which subsequently upregulates pro inflammatory genes expression. The implication of ROS in ALS pathogenesis is a growing hypothesis. In fact, several studies suggest redox dysregulation to be a primary trigger of a cascade of pathways leading to neurodegeneration (Jagaraj, Parakh, and Atkin 2021). TDP-43 pathology being shared by almost all ALS patients, it could represent a common disease initiating mechanism. Further studies are warranted in order to support these hypothesis.

Other mechanisms

Other less well characterised mechanisms that could be linked to microglial intrinsic defects are the impaired motility, reduced capacity to clear neuronal debris or impaired phagocytosis. As mentioned above, C9orf72 is a clear genetic evidence of the importance of certain ALS genes in canonical immune functions. C9orf72 is required for normal microglial function and particularly in the clearance capacity as C9orf72 deletion led to defectuous lysosomal functions in innate immune cells (ORourke et al. 2016; Plaza-Zabala, Sierra-Torre, and Sierra 2017; Corriero and Horvitz 2018; Beckers, Tharkeshwar, and Van Damme 2021). Additionally, TDP-43 has also been suggested to be associated to phagocytic functions, since microglial TARDBP knock out in mice conduct to reduced amyloid load in a model of AD, associated with drastic synapse loss (Paolicelli et al. 2017). Since a large number of ALS genes involved in the late endosomal system and lysosomal trafficking (UBQLN2, VCP, CHMP2B, TBK1, TMEM106B, OPTN, SQSTM1), are all extensively expressed in immune cells, this suggests that proteostasis may also be impaired in microglial cells and could contribute to microglial cells activation and subsequently to ALS

pathogenesis. The importance of proteostasis in ALS pathogenesis will be further detailed in the next chapter.

The ER stress apoptotic mediator marker CCAAT/enhancer binding protein (C/EBP) homologous protein (CHOP) was also shown to be colocalised to microglial cells in ALS transgenic SOD1 G93A mice and human spinal cord (Ito et al. 2009). Suggesting that ER stress is also a prominent feature of microglial activation, whether it is a consequence of other stressors or if it is a driver of the pathology in microglial cells remains elusive.

The drivers of microglial cells activation: extrinsic factors

Circulating inflammatory factors

Indeed, as aforementioned numerous inflammatory cytokines are circulating early on in ALS patients' blood and CSF as well as in ALS rodent models (Moreno-Martinez et al. 2019, Table 2-4). Some of them are thought to have anti-inflammatory effects on microglial cells while others are more pro-inflammatory. Some of these effects are detailed herein. Regarding protective functions, lentiviral vector delivery of IL-4 before onset in mutant G93A SOD1 mice increased expression of neuroprotective genes (e.g. Arg, Fizz1, Ym1) and decreased neurotoxicity-linked genes (e.g. TNF- α , IFN γ , IL-1 β). It also increased microglial proliferation while delaying disease onset. Nonetheless IL-4 delivery did not affect long term disease outcomes (Rossi et al. 2018). IL-10 expression was upregulated in the early stage of the disease; and with microglial specific lentiviral upregulation of IL-10 in CD11b+ cells, disease onset was delayed and survival was extended in mutant G93A SOD1 mice (Gravel et al. 2016). Interestingly, G-CSF treated only male mutant G93A SOD1 mice from pre-symptomatic stage until end stage had increased survival compared to non-treated mice, and was accompanied by reduction in pro-inflammatory cytokines production in monocytes and microglia (Pollari et al. 2011), suggesting that G-CSF could have protective effects in a gender specific manner.

Regarding pro-inflammatory cytokines, results are more mitigated. TNF- α levels have been reported to be increased in ALS patients and symptomatic mutant SOD1 G93A mice (Hensley et al. 2002, Table 2-4). Deletion of TNF- α in ALS G37R or G93A SOD1 mice did not change disease progression nor impact microglial activation (Geneviève Gowing et al. 2006). IL-1 β also demonstrated contradictory evidences, as deletion of IL-1 β extended survival and partially rescued MNs in G93A SOD1 mice, but not in G37R SOD1 mice (Meissner, Molawi, and Zychlinsky 2010; M.

D. Nguyen, Julien, and Rivest 2001). This may be due to differences in disease length between the two models. Surprisingly, in mutant G37R SOD1 mice treated from onset with M-CSF, accelerated disease progression was noticed, associated with decreased muscle innervation and enhanced pro-inflammatory-like microglial cells secreting TNF- α and IL-1 β (Genevieve Gowing et al. 2009). These data are particularly atypical as herein M-CSF appeared to exacerbate the disease and thus be more toxic, while previous studies showed M-CSF to be neuroprotective in other conditions such as cerebral ischemia, nerve injury and excitotoxicity (Yagihashi, Sekiya, and Suzuki 2005; Mitrasinovic et al. 2005; Lalancette-Hébert et al. 2007). Mutant G93A SOD1 mice treated with IFN γ -blocking antibody, demonstrated increased survival time with delayed disease paralysis onset without affecting disease progression; these improvements were associated with decreased pro-inflammatory genes (IL6, IL-1 β , TNF- α) and increased anti-inflammatory genes (BDNF, Arg1, ym1) expression in microglia (Garofalo et al. 2020). These findings suggest that IFN γ may be toxic early in the pathology.

Circulating factors were shown to upregulate microglial surface receptors, especially Toll-like receptors, leading to exacerbate TLR signalling in ALS disease. In ALS patients, TLR expressions are increased in ALS patients' spinal cord, supporting an increased glial reactivity (Casula et al. 2011). Moreover, GM-CSF which is highly secreted by astrocytes was shown to increase TLR4 and CD14 expression on microglia leading to intensified TLR signalling (Parajuli et al. 2012). In fact, G93A SOD1 mice lacking TLR4 showed transient improvements in hind-limb grip strength and had significantly extended survival (J. Y. Lee et al. 2015), suggesting that increased TLR signalling in microglia cells could contribute to their over-activation and drive neuroinflammation.

Altogether, circulating cytokines, chemokines circulating and other factors secreted or released from neighbouring immune cells, glial cells and MNs have an important role in microglial cells activation, as they can modulate the downstream activation pathways promoting rather pro- or anti-inflammatory response. Additionally they can also upregulate membrane receptors and thus exacerbate inflammatory response.

Protein aggregates

Aggregated control or mutant proteins can also be found in the extracellular space and could contribute to microglial activation via PPR (detailed above) (Davis, Leyns, and Holtzman 2018; Silverman et al. 2019). Extracellular misfolded SOD1 and WT or mutant TDP-43 are thought to

activate microglial cells via CD14 receptor. Interestingly, in vitro primary MNs culture exposed to extracellular human mutant G93A or G85R SOD1 did not directly cause MNs death. However, primary microglia culture exposed to these mutants displayed increased pro-inflammatory cytokines and free radicals release. Furthermore, when primary microglia were co-cultured with primary MNs and were exposed to extracellular human mutant G93A or G85R SOD1, extracellular mutant SOD1 G93A induced MNs injury (W. Zhao et al. 2010); suggesting that extracellular human mutant G93A SOD1 activate microglia into a neurotoxic phenotype which are toxic for in vitro MNs survival. Extracellular mutant G93A SOD1 and TDP-43 also seemed to induce activation of NF- κ B signalling and a subsequent inflammatory cascade as discussed previously (Frakes et al. 2014; Freeman and Ting 2016). Internalisation of extracellular TDP-43 also activated microglia NLRP3 inflammasome dependent IL-1 β and IL-18 secretion, non-canonical IL-18 processing, and neuroinflammation-linked caspase-3 activation (Frakes et al. 2014; W. Zhao et al. 2015; Leal-Lasarte et al. 2017).

Extracellular Micro-RNAs

MicroRNAs are small non-coding RNAs, which bind to the 3' untranslated region of target mRNA and direct their post-transcriptional repression. MicroRNAs have been suggested to mediate cell-to-cell communication (Xu 2013). Interestingly, TLR signalling upregulates several microRNA, and conversely many microRNA also directly target components of the TLR signalling pathways. Some microRNA have been identified to contribute to inflammatory phenotypes. Three major microRNA have been central in most research as they were upregulated upon TLR activation: they are mir-155, mir-21, mir146a (reviewed by S. R. Quinn and O'Neill 2011). Dysregulated microRNA expression was reported in the spinal cord of ALS patients and in microglia from G93A SOD1 mice (Campos-Melo et al. 2013; Parisi et al. 2013; Koval et al. 2013). Interestingly G93A SOD1 mice treated with oligonucleotide-based mir-155 inhibitor had significantly increased survival and prolonged disease duration (Koval et al. 2013). FUS and TARDBP are both linked to RNA metabolism and could play a major role in microRNA metabolism. FUS and TARDBP have both been shown to directly regulate microRNA mediated gene silencing in MNs (Santis et al. 2017; Paez-Colasante et al. 2020; Pham et al. 2020).

Interestingly several other microRNAs have been linked to inflammatory pathways activation and are thought to be involved in neuroinflammation. Mir-125b is a microglia enriched microRNA,

which is directly activated by NF- κ B and was shown to cause constitutive activation of NF- κ B by suppressing A20 expression (Parisi et al. 2016). The latter is an anti-inflammatory protein, which acts as a deubiquitinating enzyme that inhibits NF- κ B and restricts apoptosis induced by TNF α (Onizawa et al. 2015). In addition, a number of microRNAs in extracellular vesicles have been found deregulated in ALS patients biofluids (Matamala et al. 2018; Saucier et al. 2019; Dobrowolny et al. 2021), which could contribute to microglial cells activation. Thus, microRNAs may be a way of communication between cells which could contribute to microglial cell activation and lead to increased inflammation.

Direct contact with neighbouring cells

Direct contact with neighbouring cells can also induce microglial activation. Presence of T cells, dendritic cells and mast cells at the vicinity of microglial cells and MNs suggest an ongoing dialog between the innate and adaptive immune systems, which could play a major role in microglial activation (Appel, Beers, and Henkel 2010). Interestingly, activated microglia acquired properties of APC with expression of Integrin α X subunit (CD11c), Cluster of Differentiation 86 (CD86), and Intracellular adhesion molecule 1 (ICAM-1). CD86 provided costimulatory signals necessary for T cells activation and survival suggesting that these microglia interacted closely with T cells (Henkel et al. 2006; Funikov et al. 2018). Upregulation of microglial receptors have been observed in ALS patients. Toll-like receptors TLR2 and TLR4 and co receptor CD14 are found increased in ALS patients' post mortem tissues (Henkel et al. 2004; Casula et al. 2011).

Taken together, most of the direct evidences regarding microglial cells implication in ALS come from SOD1 animal models, or heterogeneous studies of ALS patients' post-mortem tissues, thus making it difficult to properly compare and understand microglial cells contribution in ALS subtypes pathogenesis. Nevertheless, identification of genes mutated in ALS, which are directly linked to immune functions, evidences of intracellular and extracellular activators, which could be shared by several ALS forms, support the idea that microglial cells are contributing to ALS pathogenesis.

4.3.2 Macrophages contribution in ALS pathogenesis

Pathology of peripheral macrophages in ALS has been more poorly described as compared to microglial cells. Reactive macrophages have been reported in ALS patients along peripheral nerves (Chiot et al. 2020). Mutant G93A SOD1 rodent models also displayed increased macrophages along the nerves, and the activation was observed pre-symptomatically and expanded from focal arrays within nerve bundles to tissue wide distribution following symptom onset (Chiu et al. 2009; Graber, Hickey, and Harris 2010; Kano et al. 2012). Interestingly, reactive macrophages at the nerves were derived from infiltrating circulating monocytes. The increased MCP-1 concomitant with increased CD68+ macrophages, suggest that MCP-1 which was possibly secreted from Schwann cells, might be a regulator for macrophages recruitment at the peripheral nerve (Tofaris et al. 2002; Kano et al. 2012). MCP-1 circulating level was also found increased in blood from ALS patients (Mitchell 2010, Kuhle 2009, Guo 2017). Interestingly, pharmacological inhibition of CSF1R which is the receptor for M-CSF and IL-34, ameliorated the disease by decreasing invading macrophages into peripheral nerves at pre symptomatic stages and by preventing microglial proliferation at the end stage of the disease (Martinez Muriana 2016). CSF1R is among others, expressed on macrophages and microglial cells and has important roles in haematopoiesis, proliferation cell survival and maturation of microglia and monocytes and control the overall immune response. A recent study from the lab, showed in the G93A SOD1 model, that replacement of peripheral mutant SOD1 macrophages by more trophic NOX2 knockout macrophages or by SOD1 WT macrophages could decrease CNS microglial cells activation, and delayed symptomatic phase of the disease (Chiot et al. 2020). This shows the importance of peripheral macrophages on CNS inflammation, and the potential interest of macrophages as therapeutic targets to modulate CNS immune reaction. Additionally, while replacement of macrophages at the pre-symptomatic stage had only an effect on symptomatic phase, replacement at the disease onset, increased ALS mice survival (Chiot et al. 2020). Transcriptomic analysis of macrophages and microglial cells over the disease course, demonstrated very distinct gene expression profiles suggesting their differential contributions in the disease. Besides mouse models, iPSC derived macrophages have been recently studied. iPSC-derived M2 macrophages from C9orf72 and sporadic patients both had immunosuppressive functions on M1 macrophages and boosted ALS Tregs differentiation from Teffectors (W. Zhao et al. 2020). This study is

interesting as it offers new perspectives for immune cell based therapy to modulate ALS patients' inflammation.

Macrophages contribution in ALS pathogenesis is still poorly described today and rely mostly on studies of mutant SOD1 mouse models. As ALS genes are ubiquitously expressed it would be reasonable to assume that similar defects to those found in microglia could be observed in macrophages. However, the study from Chiot et al., demonstrated that the two cell types responded differentially in the disease course, thus suggesting that different pathomechanisms were involved. In addition, different ALS linked genes may also have different importance in peripheral immune cells and thus further studies are necessary to better understand how peripheral macrophages are contributing to ALS pathogenesis in these ALS subtypes.

4.4 A wider implication of immune cells subtypes in ALS pathogenesis

Besides microglia and macrophages there is ample evidence that other immune cells from the innate and adaptive immune systems play an important role in the pathogenesis of ALS. Although number of different immune cells have been observed at the vicinity of the degenerating cells such as dendritic cells, mast cell, natural killers or T cells (T. Kawamata et al. 1992b; Engelhardt, Tajti, and Appel 1993; Henkel et al. 2004; C. Chen 2010; Skaper, Facci, and Giusti 2014; Garofalo et al. 2020), there is almost no evidences that these cells are responders or actors of the pathology. The direct known effect from other immune cells are only from T cells. In fact, particularly, in the past few years a subpopulation of T lymphocytes, the CD4+CD25hiFoxP3+ regulatory T lymphocytes (Tregs), have gained in importance. In the ALS G93A SOD1 mouse model, the number of Tregs were increased during the early slow progressing phase, and decreased during the fast disease progressing phase suggesting a protective role in the disease (Beers et al. 2008). Additionally, passive transfer of WT CD4+ T cells into G93A SOD1 ALS mice lacking functional T lymphocytes lengthened disease duration and prolonged survival (Banerjee et al. 2008; Chiu et al. 2008; Beers et al. 2008; Beers, Henkel, et al. 2011). In ALS patients Tregs deregulation was also observed and will be discussed in the next section.

Overall, it seems that the other immune cells are also contributing to ALS pathogenesis. However, their exact contribution remains poorly understood as no studies have tried to directly modulate these cells in animal models to measure their implication in ALS disease pathogenesis. Moreover, similarly to what has already been discussed previously, most of the studies which modulated Tregs in vivo were done on SOD1 animal models and thus the contribution of Tregs in other ALS subtypes remain to be characterised.

4.5 Human peripheral immune cells reveal increased inflammatory profiles

In recent years, the growing interest in the inflammatory role in ALS has led to characterisation and transcriptomic studies of circulating immune cells in patients. Interestingly, studies have shown altered subtype constitutions, functions and gene expressions in ALS monocytes compared to healthy controls (Lam et al. 2016; Zondler et al. 2016; W. Zhao et al. 2017). Firstly, the subtype composition was shown to be different in ALS patients and pre-symptomatic ALS patients compared to controls; with increased classical (CD14⁺⁺ CD16⁻) monocytes and a decreased non-classical monocytes (CD14⁺ CD16⁺⁺) in ALS patients compared to controls (Zondler et al. 2016). ALS monocytes showed an increased pro-inflammatory gene profile compared to controls (Lam et al. 2016; W. Zhao et al. 2017). The study of Zhao and collaborators, described that the most prominent genes signature include IL-1 β , IL-8, FOSB, C-X-C Motif Chemokine Ligand 1 and 2 (CXCL1, CXCL2). Moreover, a more pro-inflammatory profile was observed in rapidly progressing ALS patients compared to slow progressing ALS patients (W. Zhao et al. 2017). When ALS monocytes were derived into macrophages in culture, the latter produced more pro-inflammatory cytokines compared to healthy control monocytes, corroborating the previous findings of a pro-inflammatory skewed state of ALS circulating monocytes compared to controls (Du et al. 2020).

Besides innate immune cells, adaptive immunity has also been shown to play a pivotal role in the pathology. As previously described in the G93A SOD1 mouse model, Tregs deregulation has also been observed in ALS patients' blood. In particular, the number of Tregs was reduced and inversely correlated with disease progression rates (Beers, Henkel, et al. 2011; Henkel et al. 2013; Thonhoff, Simpson, and Appel 2018). ALS patients Tregs showed impaired suppressive function, which was

shown to be reversed after ex vivo expansion (Beers et al. 2021). Interestingly, an ongoing phase II clinical trial is ongoing with low dose IL-2 which is thought to target Tregs expansions. This trial has already demonstrated encouraging results by presence of increased Tregs cells in treated patients (Camu et al. 2020). Besides Tregs, Th2 lymphocytes were also shown to correlate with disease progression rate (Henkel et al. 2013). Lower levels of mRNA Gata3 (Th2) and FoxP3 (Tregs) were found in ALS patients compared to controls (Henkel et al. 2013).

Being able to study the transcriptomic profiles of circulating immune cells appears interesting as it could offer a better insight in deregulated cellular mechanisms specific to identified cell types. This has confirmed a direct implication of some immune cells in the pathology, rather than a second reaction to degenerating neuronal cells. Unfortunately, CNS cells are difficult to have access to in alive ALS patients, the use of human induced pluripotent stem cells (iPSC) to generate immune cells from different ALS subtypes represents a particularly interesting tool to better characterise CNS or PNS immune cells alteration in ALS in a human context.

As a general overview of the implication of immune cells in ALS pathogenesis. Most of the direct evidences of the immune cells implication in the pathology come from the mutant SOD1 mouse models of ALS. Studies of microglial cells and macrophages demonstrated increased inflammatory profiles and they are thought to have a dual role in the pathology firstly anti-inflammatory followed by a switch into a more pro-inflammatory phenotype. Additionally the characterisation of microglia and macrophages in the G93A SOD1 mouse model during the course of the disease demonstrated that the two cell types responded differentially during the disease course suggesting that the two cell types possibly play distinct roles in ALS pathogenesis and should be modulated differentially in the pathology. Besides microglia and macrophages, increased evidences support an implication of the adaptive immune system in ALS pathology. A part from these direct observations in SOD1 ALS pathology, the fact that some other ALS mutated genes are also linked to immune functions suggests that the immune cells may also be active drivers of the pathology in other ALS subtypes. Additionally, TDP-43 aggregates were shown to directly activate immune cells. Knowing that TDP-43 pathology is shared by most of fALS and sALS cases it could be a common pathway triggering immune cells activation. Lastly, in ALS patients studies of peripheral

cells at the transcriptomic levels revealed more pro-inflammatory profiles of monocytes, as well as altered balance of Tregs populations. Recent clinical trials trying to modulate these Tregs populations have for the moment proven interesting results, and a phase II clinical trial is ongoing (Camu et al. 2020). These study are still too preliminary to identify if modulation of the Tregs population could be beneficial to all ALS subtypes, however it offers perspectives as to identifying if modulation of the peripheral immune system could be sufficient or not to act on ALS pathology.

5- Insights in shared ALS pathomechanisms between neuronal and non-neuronal cells

Pathomechanisms have been mostly studied in MNs as they are the primarily cells to degenerate in ALS. However, most of the affected pathways reported in MNs can also be shared by other non-neuronal cells, and we could speculate that the mechanisms may be similarly affected. Certainly, some pathways are more specific to MNs such as axonal transport, excitotoxicity and NMJ defects. However, some other pathways contributing to cellular homeostasis are more widely shared by other cells types such as proteostasis, mitochondrial dysfunction, calcium homeostasis or RNA metabolism (Ilieva, Polymenidou, and Cleveland 2009; Ferraiuolo et al. 2011; Peters, Ghasemi, and Brown 2015; Burk and Pasterkamp 2019; Suzuki et al. 2020) and their disruption could have different impacts depending on the cell type.

In the past years, several proteins involved in the degradation machinery and in mitochondria (Table 6) have been found mutated in ALS patients and support a wider disruption of these pathways in numerous cell types. Notably, innate immune cells including macrophages and microglia are very active cells with high cellular trafficking owing to their phagocytic clearance functions. Disturbance in the key homeostasis functioning could have important impact on their physiological functions and contribute to ALS pathogenesis.

I have here focussed on the alteration of proteostasis as a shared pathological mechanism between MNs and other non-neuronal cells, including microglia and macrophages. In the first part I will give a general overview of proteostasis and the different ALS genes which were found to be linked to this pathway. In a second, third and fourth part will be detailed respectively the unfolded protein response, ubiquitin proteasome system and autophagy pathways. In each of these pathways, I will present the evidences of alterations of these pathways in ALS pathology.

5.1 ALS and proteostasis

Proteostasis is a term used to define protein and homeostasis. It refers to the concept of integrated biological pathways which control biogenesis, folding trafficking and degradation of proteins present within and outside the cell. Proteostasis comprises a broad network of interconnected processes.

In the past few years, the concept of altered proteostasis in pathomechanisms of ALS disease has gained importance. As has been described earlier, the propensity of certain ALS mutant proteins to misfold and aggregate imply disturbances in protein folding, quality control and degradation within the cell. In line with these findings, is the presence of TDP-43 protein deposition and inclusion formation within MNs and in glial cells in almost all ALS cases. Moreover, in the past few years, identification of genes (OPTN, UBQLN2, SQSTM1, VCP, VABP, TBK1, C9orf72, Table 6) associated with proteostasis control as risk factors to develop ALS support a role of this pathway in ALS pathogenesis.

Intracellular proteolysis is a complex mechanism involving different components (Figure 14): (1) some molecular chaperones, which monitor the misfolded proteins and inhibit their toxicity by refolding or sending them for degradation; (2) a protein quality control system operating via the endoplasmic reticulum associated protein degradation (ERAD) machinery which eliminates misfolded or unassembled proteins produced during the folding process at the ER (Nassif et al. 2010). (3) The Ubiquitin Proteasome System (UPS), which tag misfolded proteins with ubiquitin molecules and send them for degradation via the proteasome. (4) the autophagy lysosome pathway in which aberrant proteins are segregated into double membrane vesicle forming autophagosome, which fuses with lysosome to form autolysosome leading to the degradation of the content. This degradation pathway is divided into several sub-pathways including chaperone-mediated autophagy (CMA), macroautophagy and microautophagy. These several pathways of protein degradation are interdependent in cells and act synergically to maintain cellular proteostasis.

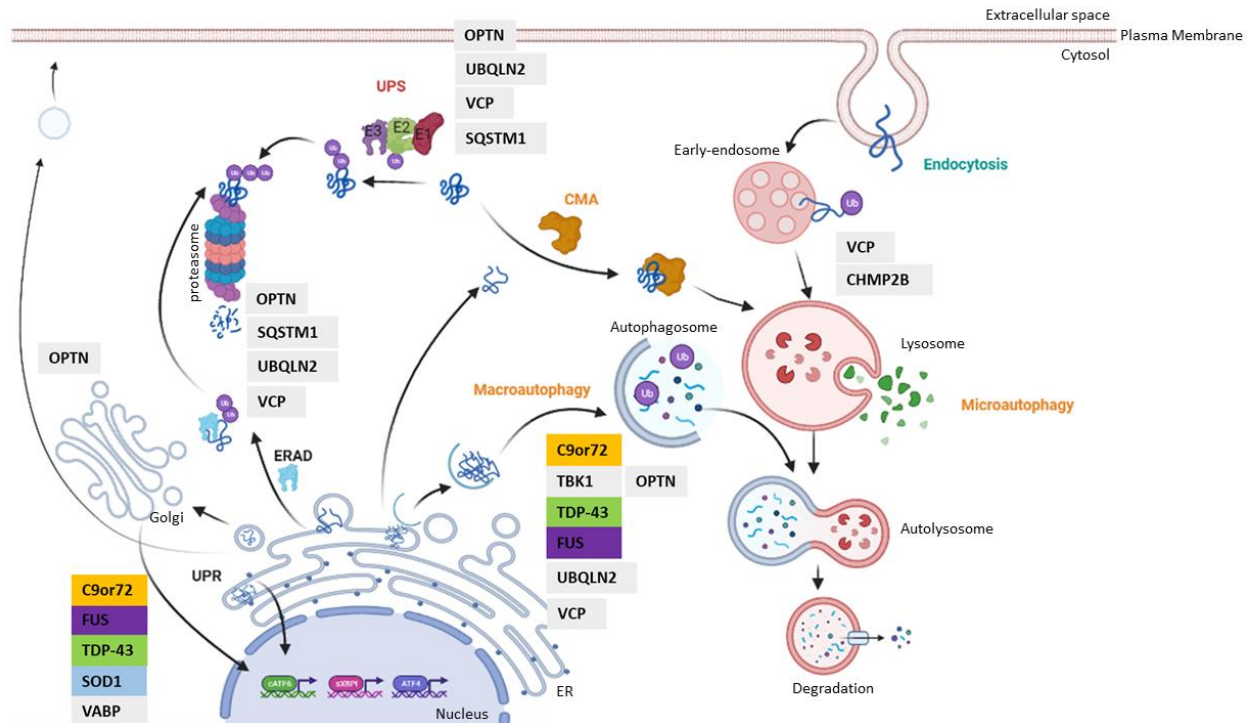


Figure 14 Schema recapitulating the different protein degradation pathways involved in proteostasis. Are also represented on the figure some ALS mutated genes, which were shown to be implicated in several of these proteostasis pathways. Three major pathways are described herein: the ubiquitin-proteasome system (UPS, in red), the endocytosis pathway (in blue) and the autophagy pathway (in orange) subdivided into microautophagy, macroautophagy and chaperone mediated autophagy (CMA). The unfolded protein response (UPR) is mediated by the endoplasmic reticulum system (ER) and lead to protein degradation via either UPS or CMA pathways. UPS: Ubiquitin proteasome system; CMA: chaperone-mediated autophagy; UPR: unfolded protein response; ERAD: endoplasmic reticulum associated protein degradation; Figure generated with Biorenders

5.2 Endoplasmic reticulum stress, Unfolded protein response (UPR) and endoplasmic reticulum associated protein degradation (ERAD)

5.2.1 General presentation of UPR and ERAD

Endoplasmic reticulum (ER) stress has been suggested as a main actor in ALS pathology, mainly due to its pleiotropic functions in cellular homeostasis (Tobisawa et al. 2003; Nagata et al. 2007; Saxena, Cabuy, and Caroni 2009). In fact, ER is composed of endomembranes, which mediate protein synthesis, post-translational processing and folding of new membrane and secretory proteins. In addition, it plays an important role in intracellular transport for RNA, secretory factors, proteins and ions. ER is also a large calcium storage, playing a critical role in cellular calcium signalling. It interacts in the ER Mitochondria calcium cycle (ERMCC), coupling energy metabolism in mitochondria and protein processing in the ER to synaptic activity in neurons (Lautenschlaeger,

Prell, and Grosskreutz 2012). Disturbance in the ER environment by cellular stress conditions including glucose deprivation, oxidative stress, elevated secretory protein synthesis, accumulation of unfolded or misfolded proteins or changes in calcium homeostasis, lead to accumulation of unfolded or misfolded proteins in the ER lumen and activation of ER stress machinery (Kaufman 2002; Medinas et al. 2018). The latter triggers unfolded protein response (UPR) implying activation of three major transmembrane protein of the ER: the PKR-like ER protein kinase (PERK), the activation transcription factor 6 (ATF6) and the inositol requiring enzyme 1 (IRE-1) which together aim at restoring balance between folding capacity of the ER and the folding demand (Figure 15). To do so, the ER decreases the folding demand by downregulating transcription of genes and translation ; while it increases clearance of misfolded protein via ER-associated degradation (ERAD) machinery (Hetz and Papa 2018; Nam and Jeon 2019). ERAD is a central component of protein homeostasis as it serves as a quality-control mechanism that can eliminate terminally misfolded or unassembled polypeptides whose propensity for aggregation may cause toxicity. It also acts as a regulator for enzymes and signalling molecules levels in response to disrupted homeostasis (Brodsky and Wojcikiewicz 2009; Christianson and Ye 2014). Misfolded proteins can be eliminated either via LC3 mediated autophagy or via ubiquitin-proteasome system, which both will be detailed in the next session (Ron and Walter 2007; Christianson and Ye 2014). Several molecular chaperones are involved in these responses such as ER chaperone glucose regulated protein 78 (GRP78/BiP), calreticulin, endoplasmic reticulum chaperone protein disulfide isomerase (PDI/PDIA1). As ER is a multifunctional organelle implied in many cellular functions, its perturbation usually does not remain localised to the ER but spread through the secretory pathway, the cell and the organism. Moreover, high levels of chronic ER stress may result in apoptosis (Nam and Jeon 2019).

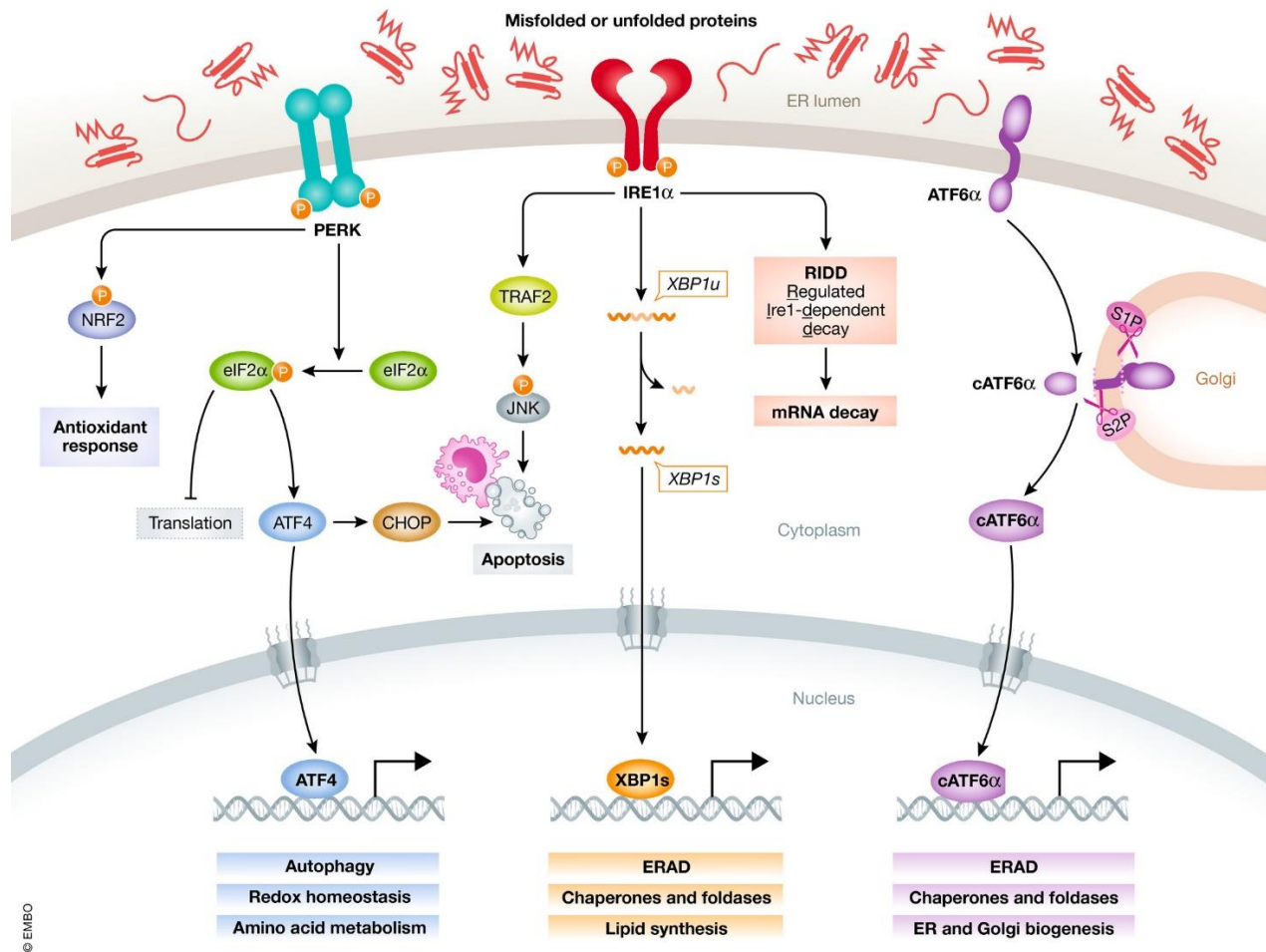


Figure 15 Schematic representation of the unfolded protein response (UPR) in the endoplasmic reticulum (ER) and the ER-associated degradation (ERAD). The UPR is a stress response linked to ER overload by unfolded or misfolded proteins, which aim at restoring proteostasis. Three stress sensors located at the ER membranes are governing the UPR. They are: activating transcription factor 6 (ATF6), inositol-requiring protein 1 (IRE1), and protein kinase RNA (PKR)-like ER kinase (PERK). In homeostatic conditions the sensors are inactivated via direct binding of chaperone, binding immunoglobulin protein (BiP) at the luminal domain. Upon ER stress condition, BiP are released and conduct to activation of the UPR. The ERAD is a conserved protein degradation machinery of the ER which removes unfolded or misfolded proteins via the ubiquitin proteasome system (UPS) From T. Zhang et al. 2020

5.2.2 Evidences of ER perturbation in ALS

In ALS patients and animal ALS models, several lines of evidence suggest ER disturbance, which may lead to altered cellular homeostasis and possible cell death. Observation of post-mortem spinal cords from sALS cases revealed altered rough ER (rER), with irregularly distended morphology and detachment of the ribosomes in anterior horn MNs displaying shrunken phenotype. In MNs with central chromatolysis, rER appeared fragmented but retained their width and had normal ribosomal attachment (Oyanagi et al. 2008). Dilated predominantly normal appearing ER contained accumulation of amorphous and granular material, possibly being

misfolded or unfolded proteins (Sasaki 2010). In addition to these structural defects, expression of components of the UPR pathways were found increased in the spinal cord of patients with sALS and fALS (Nagata et al. 2007; Ito et al. 2009; Sasaki 2010; Medinas et al. 2018). Protein biomarker screening was done on sALS patients Peripheral blood mononuclear cells (PBMC) and revealed three major markers associated with the disease which could discriminate against ALS patients' from healthy control with an accuracy of 98%. ER stress protein 72 (ERp72), a luminal protein of the ER, was part of these three markers (Nardo et al. 2011). Suggesting that ER alterations may be not only in MNs but also in other non-neuronal cells, including immune cells.

In G93A SOD1 mouse models, studies revealed accumulation of ER stress markers in regions where neurodegeneration underwent but not in non-affected regions. Additionally, mutant SOD1 protein and to a lesser extent WT-SOD1 accumulated in the ER (Kikuchi et al. 2006). Interestingly, a recent study has shown that WT-SOD1 aggregation increases with aging and that this aggregation was favoured at the ER. Additionally, mice expressing the human WT-SOD1 exposed to chronic ER stress presented accumulation of WT-SOD1 aggregates in the spinal cord MNs at 4 and 16 months of age (Medinas et al. 2018). These findings were supported by the occurrence of WT-SOD1 aggregates significantly increased in the lumbar spinal cord of sALS patients compared to control subjects (Ito et al. 2009; Medinas et al. 2018).

IPSC modelling has also provided some insight into defects in proteostasis and ER stress. Transcriptomic analysis of iPSC derived SOD1 A4V mutated MNs showed elevated expression of several ER stress markers supporting an active UPR (Kiskinis et al. 2014). Nonetheless, control MNs also expressed markers of UPR, albeit at a lower level. This suggests a possible increased sensitivity of ALS MNs to ER stress and could partly explain the selective vulnerability of these cells in ALS pathology. In accordance with these results, MNs subtype selective ER stress vulnerability was demonstrated in three mouse models (high-expression G93A SOD1-fast mice, low-expression G93A SOD1-slow mice and G85R SOD1 mice) of fALS. Vulnerable MNs had increased gradual upregulation of ER stress markers from birth on (Saxena, Cabuy, and Caroni 2009).

In spite of mutant SOD1 models, iPSC derived C9orf72 mutated MNs displayed elevated ER calcium with an increase of up to twofold in cytoplasmic fluorescence intensity when artificially triggering ER calcium depletion. Abnormal elevation of ER calcium can lead to ER stress. In fact, UPR GRP78 chaperone protein level was markedly increased in iPSC-C9orf72 MNs compared to control MNs

(Dafinca et al. 2016) this was also confirmed in another study (Kiskinis et al. 2014). In fact, the decreased survival of C9orf72 iPSC derived MNs may be attributable to ER stress in combination with impaired calcium balance and abnormal mitochondrial function.

A study has also generated Vesicle-associated membrane protein-associated protein B (VAPB) mutated iPSC-derived MNs, which developed chronic ER stress, resulting in secondary oxidative stress, mitochondria impairment, synaptic pathology and cell death (Hall et al. 2017).

In ALS, several independent molecular triggers are thought to adversely affect the ER, which are directly linked to the mutated ALS proteins. The Table 6 shows how different mutant proteins can directly affect the ER machinery by interacting with different components of the UPR or ERAD pathway. Moreover, VAPB is directly involved in ER functioning and mutations in this gene has been reported in a small proportion of ALS patients (Nishimura et al. 2004). Mutation in the gene can lead to its impaired normal functioning and conduct to ER stress (Cadoni et al. 2020).

Table 9 Evidences of direct interaction of mutant proteins with ER components

| Molecular triggers | Location or interaction with ER components | Consequences | References |
|-------------------------|---|--|--|
| Mutant SOD1 G93A | ER lumen | Prevent normal function of essential chaperone | Kikuchi et al. 2006; Urushitani et al. 2008 |
| | Direct interaction with Derlin-1 (ERAD component) | Inhibition of the mSOD1-Derlin interaction with small molecules In vivo: delayed disease onset increased survival In vitro: L144FVx iPSC-MNs increased survival but not in G93S iPSC-MNs | Kikuchi et al. 2006; Tsuburaya et al. 2018 |
| Mutant TDP-43 | Mislocalisation in cytoplasm | Induce ER stress and fragmentation of Golgi apparatus | Walker et al. 2013; Dafinca, Barbagallo, and Talbot 2021 |
| Mutant FUS | Interaction with PDI chaperone | Trigger ER stress | Farg et al. 2012 |
| C9orf72 DPR | GA | ER stress, neurotoxicity | Y.-J. Zhang et al. 2014; May et al. 2014b |
| VAPB | Normal function: Interaction with ATF6 and IRE-1 | Mutation in this gene can interrupt UPR response | Hirano 2008; Vinay Kumar et al. 2014 |

This shows us that the majority of proteins involved in ALS may have a function in ER stress; however, other more global mechanisms such as disruption of calcium balance could also explain the presence of ER stress in patients not carrying mutations in these genes. Taken together, all current data suggest that ER stress and UPR response are common pathological mechanism between several ALS forms and could be implicated in MNs vulnerability, but also in other non-neuronal cells ER stress and contribute to ALS pathogenesis.

5.3 Ubiquitin Proteasome System (UPS)

5.3.1 General presentation of UPS

The Ubiquitin Proteasome System (UPS) is a proteolytic mechanism whereby the conjugation of ubiquitin (Ub on the figure) to specific substrates, leads to their degradation via the proteasome (Figure 14). The substrate ubiquitination is an ATP-dependent enzymatic process involving ubiquitin-activating (E1) enzyme, ubiquitin-conjugating (E2) enzyme, and ubiquitin-ligase (E3), to tightly coordinate protein turnover in the cell (Nandi et al. 2006). Substrate proteins are first recognised by E3 ligase protein which recruits E2-enzymes charged with ubiquitin. Upon ubiquitination the substrate is translocated to the proteasome, composed of a core (20S) particle with proteolytic sites and a 19S regulatory particle governing access to the core (Clague and Urbé 2010). Normal substrates of the UPS pathway include misfolded proteins, and short-lived proteins (Ravid and Hochstrasser 2008). For the ERAD system, polyubiquitination of the substrate takes place at the ER membrane. E1 first activates ubiquitin, ensuing ubiquitin-conjugating enzyme E2 acting with E3 ligase to transfer ubiquitin on the desired substrate. Two major E3 ligases are well conserved through evolution and are responsible for most of the ubiquitination in ERAD (Hrd1p and Doa10p) (Christianson and Ye 2014). In mammals, a number of ERAD related E3 ligases can be recruited to deal with folding crises at the ER (such as GP78) which is usually linked to large or prolonged variations in the cell such as change in calcium or redox homeostasis, accumulation of misfolded protein or else (Mehnert, Sommer, and Jarosch 2010).

UPS plays a vital role in a myriad of cellular functions, englobing temporal and spatial activation of key proteins regulating cell cycle progression, apoptosis transcription and development. Additionally UPS is involved in immune functions, via generation of the majority of the peptides

presented by the MHC-II as well as clearance of aberrant proteins in the cellular environment. UPS is also involved in DNA repair, membrane transport, chromatin remodelling, transcription and signalling pathways (Bhat, Truax, and Greer 2010). The major involvement of UPS in key cellular processes as well as protein quality control places it in a sensitive position. This holds particularly true in the cases of ND in which accumulation of misfolded and damaged proteins is a major hallmark (Tydlacka et al. 2008; Kikis, Gidalevitz, and Morimoto 2010; Ruegsegger and Saxena 2016).

5.3.2 Evidences of UPR perturbation in ALS

In ALS, multiple lines of evidence support defects in the UPS pathway. The most evident being the accumulation of ubiquitin positive inclusions in neuronal and glial cells of sALS and fALS as a major hallmark of the pathology (Leigh et al. 1991; M. Neumann et al. 2006; Mackenzie, Frick, and Neumann 2014; 2014; Ruegsegger and Saxena 2016). As mentioned previously, TDP-43 accumulation is a prominent hallmark of ALS disease and its mislocalisation causes phosphorylation and aggregation of TDP-43, which is targeted for ubiquitination (Kametani et al. 2016). TDP-43 accumulation has been classified into six classes ranging from soluble to undegradable aggregates. The clearance via UPS or autophagy is thought to depend on the solubility of the oligomers (Casella et al. 2017). Moreover, a recent study showed that overexpression of TDP-43 impairs UPS in mammalian cells and *Drosophila* models of TDP-43 proteinopathies (P. Wang et al. 2019; S. Lee et al. 2020). The toxicity was mediated by accumulation of TDP-43 in neuronal cells leading to decreased proteasome activity (S. Lee et al. 2020). These results are supported by a study showing that cells treated with protease inhibitors demonstrated an accumulation of ubiquitinated and insoluble TDP-43 particles, suggesting that the UPS rather than the autophagy pathway is predominantly involved in the degradation of TDP-43 aggregates (Araki et al. 2014). Besides TDP-43, it has been shown that numerous different ALS-linked proteins interact in a way or another with the proteasomal system (Webster et al. 2017; Maurel et al. 2018; Le Guerroué and Youle 2021).

Misfolded SOD1 protein is generally tagged by ubiquitin and is supposed to be targeted for degradation by the proteasome system. ALS animal models and patient's post mortem studies suggest that mutant SOD1 hampers proper proteasomal function and autophagy and escape degradation, as suggested by increased number of autophagosomes in MNs of the spinal cord (Bendotti et al. 2012). This escape further lead to exert toxic effect of mutant SOD1 on the

degradation machinery (Hyun et al. 2003; Cheroni et al. 2009; Bendotti et al. 2012). The dysfunction of the UPS in ALS was also demonstrated by crossing SOD1 G93A mice with transgenic mice expressing UbG76V-GFP (a fluorescent-tagged reporter substrate of the UPS) (Cheroni et al. 2009). In these mice, increased accumulation of the UbG76V-GFP selectively in a few spinal and cranial MNs was observed from symptomatic stages of the disease, indicative of UPS impairment (Cheroni et al. 2009).

In C9orf72 mutated patients, the UPS pathway is also thought to be altered as E2 genes (UBE2I, UBE2QI, UBE2E1, and UBE2N) displayed significant dysregulation (Serpente et al. 2015). Moreover, C9orf72 protein long isoform was shown to form a complex with Smith-Magenis Syndrome Chromosome Region, Candidate 8 (SMCR8), and was shown to interact amongst others with numerous ubiquitin-related proteins (reviewed by Goodier et al. 2020).

Altogether, it would appear that defects of major ALS mutations converge to a defect of the UPS system even if the initial function of the protein is not directly related to this system.

Genetic evidence also supports an implication of the UPS system in ALS pathogenesis, as some ALS-linked gene mutations affect proteins that directly interact with the UPS.

- Ubiquilin-like protein ubiquilin 2 (UBQLN2), is a shuttle protein implicated in the proteasome mediated protein degradation, and was found mutated in a subset of ALS and FTD patients (H.-X. Deng et al. 2011; Renaud et al. 2019). UBQLN2 was also shown to interact with Hsp70 type chaperones. Mice knock-in for mutated UBQLN2 showed defective chaperone binding, impaired aggregate clearance and cognitive deficits (Hjerpe et al. 2016).
- The valosin-containing protein (VCP) was also shown to be mutated in a small proportion of ALS patients (J. O. Johnson et al. 2010; Abramzon et al. 2012). p97/VCP is a member of the large ATPase family protein associated with diverse cellular activities essential for maturation of ubiquitin containing autophagosomes. It participates in a wide range of cellular functions such as in ERAD by providing the energy source driving dislocation of the polypeptide targeted for degradation from the ER (Christianson and Ye 2014). P97/VCP also serve as ubiquitin selective chaperone using the energy from ATP hydrolysis to segregate its ubiquitinated substrate from immobile structures. VCP has a central role in cellular proteostasis as in cells and animals lacking VCP or expressing mutant VCP, the early

autophagosome vacuoles fail to mature into autolysosomes, resulting in the accumulation of non-degradative autophagosomes, as well as mitochondrial abnormalities (Ju et al. 2009; Nalbandian et al. 2012).

In conclusion, UPS plays a critical role in the degradation of a myriad of cellular proteins, especially misfolded proteins. In ALS, genetic mutations as well as environmental factors promote the formation of misfolded proteins which accumulate in the cytoplasm of neuronal and glial cells. The UPS overload and steric hindrance may participate to cellular vulnerability in MNs leading subsequently to their death, and in other non-neuronal cells overload may lead to cell activation such as in the cases of innate immune cells.

5.4 Autophagy

5.4.1 General presentation of autophagy pathway

Autophagy is a predominant lysosomal dependent protein degradation process encompassing three major ways: macroautophagy, microautophagy, CMA (Figure 14). Autophagy usually helps removal of long-lived proteins, aggregate prone pathogenic proteins or damaged organelles. Macroautophagy is associated with bulk degradation of cytoplasmic components via formation of autophagosomes, which can directly fuse with late endosomes or lysosomes to form autolysosomes. This pathway is mainly involved in the removal of mutant misfolded proteins in several ND including ALS (Ciechanover and Brundin 2003; Parzych and Klionsky 2014). Microautophagy is characterised by direct uptake and degradation of cytoplasmic components by lysosomes (Mijaljica, Prescott, and Devenish 2011; Sahu et al. 2011). CMA is a way of degradation implying recognition of specific sequences on the substrate that help binding of Hsp70 followed by recognition of the substrate-chaperone complex by LAMP2A. The complex is unfolded and taken across lysosomal membranes (Cuervo and Wong 2014). Due to the central role in cellular protein homeostasis impairment of lysosomal process can lead to cellular toxicity and possible neuronal degeneration (Ruegsegger and Saxena 2016; Sonninen et al. 2020).

5.4.2 Evidences of impaired autophagy in ALS

Autophagy deregulation has been observed in ALS patients tissues mainly due to presence of p62 positive and ubiquitin positive inclusions (Al-Sarraj et al. 2011; Cooper-Knock et al. 2012; Mackenzie, Frick, and Neumann 2014). ALS genes are shown to have direct relations with the autophagy system.

Although little is known about the function of C9orf72 protein, bioinformatic studies predicted that the major structural domain of the protein is a DENN domain protein. DENN proteins are best characterised as regulators of intracellular membrane traffic via GEF for Rab GTPases (D. Zhang et al. 2012; Levine et al. 2013), thus suggesting a prominent role of C9orf72 in membrane trafficking, with evidence of its implication in autophagic and lysosomal systems (Farg et al. 2014; Sellier et al. 2016a; Webster et al. 2016). Various studies have showed that C9orf72 complexes formed with SMCR8 and WD Repeat domain 41 (WDR41) bind Rabs and have important roles in autophagy, initiation of autophagosome formation, endosomal and lysosomal pathways (Farg et al. 2014; Amick, Roczniak-Ferguson, and Ferguson 2016; Sullivan et al. 2016; M. Yang et al. 2016; Ugolino et al. 2016; Webster et al. 2018; Balendra and Isaacs 2018; Shi et al. 2018; Leskelä et al. 2019). Reduction of c9orf72 expression in cell lines and primary neurons decreased autophagy and caused p62 positive inclusions (Sellier et al. 2016a; Webster et al. 2016; ORourke et al. 2016). SMRC8, one of the interactors of C9orf72, is phosphorylated by TBK1 suggesting a common implication in the autophagy pathway between these different ALS proteins.

TDP-43, was also shown to interfere with the autophagy system. As mentioned above, large insoluble TDP-43 aggregates require autophagic clearance (Scotter, Chen, and Shaw 2015). In fact, the protein itself seems to play a role in the system, as RNAi knockdown of TDP-43 in cell lines showed deregulation of Autophagy related protein 7 (ATG7) (Bose, Huang, and Shen 2011). The latter is an integral constituent of the autophagy machinery. Interestingly, genetic upregulation of ATG7 in mutant TDP-43 mice attenuated motor dysfunction associated with TDP-43 depletion. Suggesting that TDP-43 has a direct role on ATG7 and the autophagy system (Donde et al. 2020). Additionally, compounds stimulating autophagy improved TDP-43 clearance and enhanced survival of primary murine neurons and human stem cells derived neurons (Barmada et al. 2014). Evidences of FUS implication in autophagy come from FUS mutated patients' tissues which demonstrated increased microtubule-associated protein 1A/1B-light chain 3 (LC3/ MAP1LC3)

positive vesicles in MNs of the spinal cord compared to controls (Matsuoka et al. 2011; Soo et al. 2015). LC3 is a central player of the autophagy pathway involved in substrate selection and autophagosome biogenesis. Other evidences come from study in which the mutant FUS protein expression in neuronal cells led to accumulation of ubiquitinated proteins p62 and autophagy markers NBR1; additionally the cells had impaired autophagosome formation. Interestingly, overexpression of Ras-related protein 1 (Rab1), a protein involved in intracellular membrane trafficking, rescued these defects (Zoppino et al. 2010; Soo et al. 2015), thus suggesting that mutant FUS inhibits Rab1 activity and consequently possibly alter intracellular trafficking.

Genetic evidence supports defects in autophagic clearance as a pathomechanism driving ALS disease.

- *SQSTM1* mutations have been described in 1-3% of fALS patients (Fecto et al. 2011; Teyssou et al. 2013). *SQSTM1* gene encodes the protein p62 involved in various cellular processes such as transcription, degradation by UPS, autophagy and apoptosis. As p62 is an ubiquitin-binding protein, it may be involved in the UPS, yet it is better known for its role in autophagy. P62 is known to interact with LC3 for selective autophagy, and mutations in the binding domain have been identified in ALS patients (Goode et al. 2016). Knock-down of the ortholog zebrafish *sqstm1* gene led to increased of the Mammalian target of rapamycin complex 1 (mTORC1) level, a downstream effector of p62, subsequently leading to locomotion defects and axonal abnormalities. The phenotype could be rescued by overexpression of wild type *sqstm1* gene (Lattante et al. 2015). Autophagy is also responsible for p62 degradation, and thus impaired autophagy is usually accompanied by massive p62 accumulation and aggregates positive for ubiquitin and p62 (Komatsu and Ichimura 2010).
- *OPTN* gene encoding optineurin has also been linked to ALS (Millecamps et al. 2011; Véronique V. Belzil et al. 2011). Optineurin is involved in Golgi apparatus maintenance, and vesicular and membrane trafficking. Similarly to p62 it contains ubiquitin-binding domain and LC3 interacting regions, suggesting an involvement in UPS and autophagy. TBK1 mediates phosphorylation of optineurin, further driving autophagy (Richter et al. 2016). Optineurin also regulates autophagy through its interaction with myosin VI, promoting fusion of autophagosomes with lysosomes (Tumbarello et al. 2012). Mutations in the

ubiquitin-binding domain lead to compromised autophagosomes maturation, autophagy and clearance of inclusion bodies (Sirohi and Swarup 2016).

- TBK1 as presented earlier has also been linked with a subset of ALS patients (Freischmidt et al. 2017). This protein is involved in two pathomechanisms in ALS, inflammation and autophagy (Ahmad et al. 2016; Maurel et al. 2018). TBK1 is a kinase and thus has a role in phosphorylation of several factors. Most of the mutated TBK1 variants are thought to mediate toxicity via haploinsufficiency rather than a GOF (Ahmad et al. 2016). In autophagy *TBK1* mutations were shown to prevent phosphorylation of autophagic receptors including OPTN, and p62 and thus failed to mediate engulfment of polyubiquitinated autophagosomes (Matsumoto et al. 2015). In inflammation, TBK1 regulates the expression of inflammation mediators (IL-6, TNF- α , IFN- β) (Ahmad et al. 2016). More generally a myriad of different interactors of TBK1 have been reported which are linked to immune response, inflammation, autophagy proliferation and growth, as well as the insulin signalling pathway (Helgason, Phung, and Dueber 2013).
- UBQLN2 and VCP, which were described in the previous section, were also shown to have an implication in the autophagy pathway. UBQLN2 predominantly via its interaction with LC3 and thus the delivery of cargo to autophagosomes (N'Diaye et al. 2008; Rothenberg et al. 2010; Wu et al. 2020). A recent study also identified UBQLN2 role in vacuolar acidification by the ATPase pump, necessary for autophagosome maturation. Knock-out of UBQLN2 in cells and mice lead to decreased ATPase H⁺ Transporting V1 Subunit G1 (ATP6V1G1), one subunit of the ATPase pump (Wu et al. 2020). VCP on the other hand, has for a long time been associated with the autophagic system (Krick et al. 2010; Ritz et al. 2011; Ramanathan and Ye 2012; Buchan et al. 2013; Papadopoulos et al. 2017). In a recent study using cellular models, VCP was shown to translocate in lysosomes and interact with several mediators of which, deubiquitinating enzymes. Together they remove ubiquitin conjugates to promote autophagosome formation and efficient clearance of the damaged organelles (Papadopoulos et al. 2017). Furthermore, mutation in the *VCP* gene attenuated lysosomal clearance and damaged lysosomes accumulated in mouse embryonic fibroblasts (Papadopoulos et al. 2017).

In conclusion, the UPS and autophagy systems work in synergy to maintain cell balance. There is much evidence to support both system's involvement in the pathogenesis of ALS, although the exact mechanisms have not yet been identified.

Taken together, proteostasis is as a finely regulated complex of different systems that tightly interact with each other. Protein aggregates is a prominent hallmark of ALS pathology in neuronal cells and could be linked to defectuous protein degradation machinery, but as a vicious circle, overloading of protein aggregates could also contribute to the impairment of the degradation machinery. To date most of the researches regarding proteostasis defects in ALS have been conducted in MNs. However, immune cells necessitate important degradation functions mainly due to their phagocytic activities. The observed proteostasis dysregulation could have major effects on ALS microglial cells and macrophages activation. Moreover, numerous genes which have been found mutated in ALS disease are directly linked to the degradation machinery thus reinforcing the importance of proteostasis in ALS pathogenesis in neuronal cells but also importantly in non-neuronal cells.

6- Induced pluripotent stem cells to study ALS

Advances in recent years in human stem cell biology have opened up new opportunities to develop human disease-specific cell types that allow us to better understand and explore the mechanisms that underlie disease pathogenesis. In particular, the exciting technology which allow to reprogram human somatic cells into induced pluripotent stem cells (iPSC) gives a considerable advantage in generating iPSC from patients with specific genetic signatures, but even more interestingly to generate iPSC from sporadic patients which heretofore were not studyable in animal models. A further revolution in the field was the discovery of CRISPR-cas9 technology. The adjunction of both technologies makes it possible to correct the mutation or add a mutation while keeping the same genetic background and thus offers new avenues in understanding the exact role of a mutation in pathological phenotype.

The particular advantage of using iPSC to study ND is the possibility to generate cells that are not accessible in alive patients. Thanks to this, many questions can be asked in a human context and

this implies in particular the understanding of inherent defects in the different cell types which can be mediated by the genetic defect, of the specific vulnerabilities of certain neuronal types in relation to others in the given pathology as well as the interactions between different cell types. The first protocols that appeared allowed the generation of neuronal types. Nowadays, other protocols allowing the generation of different cell types such as glial, myoblasts or else have emerged. This renders possible the study of intrinsic defects in these cell types as well as their mediated trophicity/toxicity by conducting co-cultures. More recently, 3D cultures like microfluidics or brain organoids, are starting to arise allowing more integrated studies of specific neuronal circuits. In this chapter I will first detail the protocols which permitted the obtention of iPSC derived MNs and the observed pathological hallmarks of ALS disease in these cells. In a second part I will detailed the current knowledge regarding differentiation of iPSC cells into innate immune cells.

6.1 iPSC derived MNs harbour ALS pathological hallmarks

6.1.1. Protocols to generate iPSC derived MNs

The first protocols that appeared to generate MNs from iPSC only made it possible to obtain generic neurons with very poor purity. With technology advances, many well characterised MNs subtypes can now be generated, which offer the unique abilities to capture some key features of ALS pathogenesis and underscore their potential roles in drug discovery.

Since 2007 a dozen of different protocols to generate lower MNs having their own timings (ranging from 28-130 days of differentiation) or purity (ranging from 20-95% of MNs) arose (Sances et al. 2016). Albeit these differences all the protocols are based on three major steps which are the neural induction by dual SMAD inhibition followed by caudal and ventral patterning via exposition to retinoic acid and SHH and lastly MNs maturation using specific neurotrophic factors (Sances et al. 2016). These steps of sequential differentiation from iPSC to MNs are recapitulated in the Figure 16.

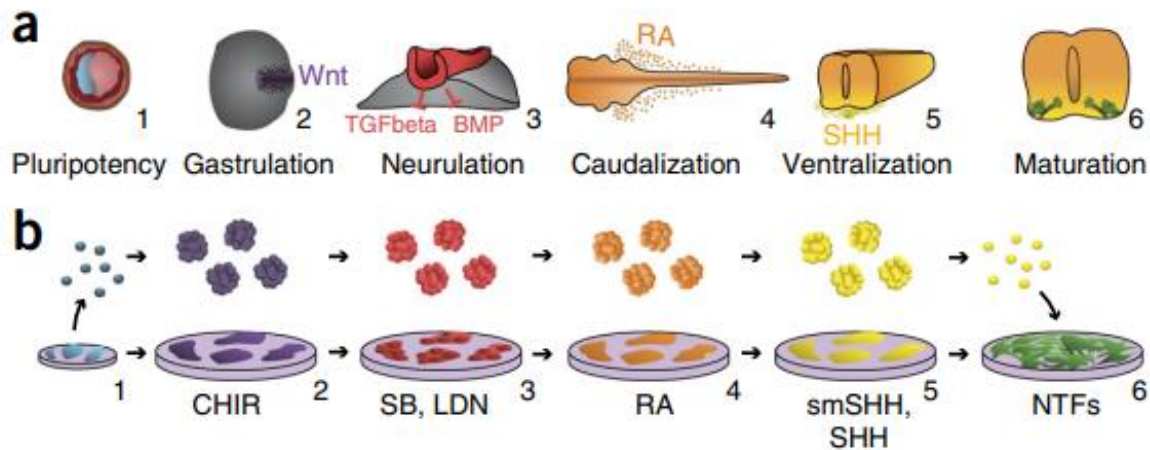


Figure 16 Schematic representation of spinal MNs differentiation steps in vitro by mimicking neurodevelopmental steps using small molecules and recombinant signalling molecules. In vivo pluripotent cells are present in the blastocyst. During gastrulation, Wnt dependent primitive streak formation (a2) is simulated in vitro using glycogen synthase kinase 3 inhibitor CHIR-99021 (CHIR) (b2). Further on, the neurulation step is induced by the use of dual SMAD inhibitors (SB431542a and LDN193189) (b3). In vivo caudal patterning is mediated by retinoic acid (RA) gradient produced by neighboring somites. The different concentrations specify hindbrain and anterior spinal cord fate. In vitro a specific concentration of RA is used to direct caudalisation of the neural ectoderm. In vivo the ventral patterning is directed by SHH gradient released by the ventral notochord. In vitro usage of small molecule SHH or recombinant SHH signaling agonists permit to recapitulate the ventralisation process. MNs progenitors thus necessitate trophic support in order to mature into functioning lower MNs. In vitro different cocktails of neurotrophic factors are used to promote maturation and survival (BDNF, GDNF, CNTF, IGF1). Sances et al., 2016

In the human embryonic spinal cord 44% of MNs are ISLET-1 positive, 31 % are HB9 positive and 25% co-express both factors (Amoroso et al. 2013). In vitro these two different factors are commonly used to identify MNs. However, different MNs subtypes are present in vivo, which are connecting distinct muscles and display distinct intrinsic properties (Stifani 2014). Regarding the protocols allowing iPSC generation of lower MNs it is often not specified which subtype of MNs are generated. Spinal MNs are arranged in distinct columns termed motor column, extending along the rostro caudal axis (Stifani 2014). Each motor column has its own gene expression and axonal projection pattern (Figure 17). Some transcription factors are specific of MNs subtypes and allow better characterisation of the MNs obtained in in vitro cultures (Stifani 2014; Mouilleau et al. 2021). The Figure 16 is presenting the organisation of the different motor column and present also the specific proteins expressed by each motor column (Stifani 2014, Figure 17). Recently, several studies have investigated the expression of HOX genes, which are involved in the identities of MNs columns along the rostral caudal axis of the spinal cord (Cave and Sockanathan 2018; Mouilleau et al. 2021). The better understanding of how these transcription factors govern MNs

identity open new perspectives to generate highly specific MNs from different spinal regions, which may be differentially affected in ALS patients.

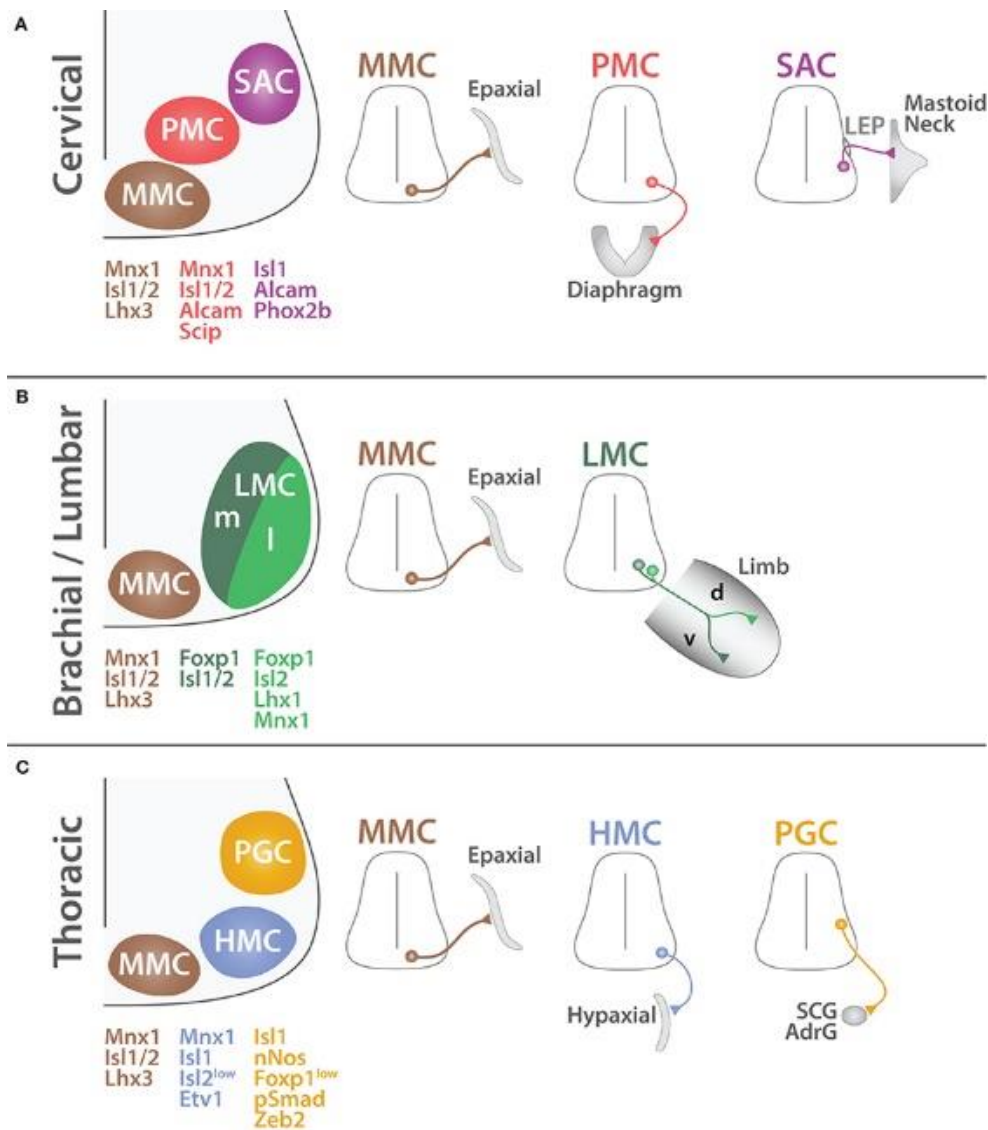


Figure 17 Organisation of the spinal MNs at different levels of the spinal cord (Cervical (A), Brachial/Lumbar (B), Thoracic (C)). Median motor column (MMC) are located medially and is present all along the rostro-caudal axis. MNs from this column are innervating axial musculature. (A) Phrenic motor column (PMC) is restricted to between cervical segment 3 to 5, MNs in this column innervate the diaphragm. Spinal accessory column (SAC) is restricted to the five first cervical segments and innervate mastoid and neck muscles. (B) Lateral motor column (LMC) is located at limb levels and innervate the latter. (C) Hypaxial motor column (HMC) is exclusively thoracic and innervate intercostal muscles. Preganglionic column (PGC) extends through the thoracic segments until the second lumbar segments (L2) and between sacral segment s2 and s4 and innervate to the sympathetic chain ganglia and chromaffin cells of the adrenal gland. Stifani et al., 2014

Taken together, owing to the diversity of protocols being established to generate MNs, in order to properly characterise and compare the cell types obtained from one protocol to another, it would be crucial to have standardised criteria to enable adequate comparisons of results between studies. Indeed, although the comprehension has advanced significantly in the past years, much remains to be determined.

6.1.2 ALS pathological hallmarks in MNs

In ALS, both upper and lower MNs are degenerating. Most of studies focused on iPSC derived spinal MNs. Although cortical MNs protocols are starting to emerge. In the past years, studies generating iPSC derived ALS MNs (ALS iPSC-MNS) have revealed disease-specific phenotypes, validating the use of cellular models to explore the molecular basis of ALS and to develop new screening platforms for drug development.

iPSC derived familial ALS MNs survival

The most prominent hallmark of ALS MNs are their death along disease. In in vitro cultures, ALS MNs degeneration and death are not observed for all ALS mutants and usually represent a tardive event, which may depend also on culture conditions. Several studies in SOD1 mutant iPSC-MNs reported decrease survival of MNs over time (Bhinge et al. 2017; Imamura et al. 2017; Kiskinis et al. 2014). In TDP-43 mutant iPSC-MNs, some studies reported diminished MNs survival over time while others studying the same mutation did not reported these observations (Devlin et al. 2015; Fujimori et al. 2018). Some other studies using TDP-43 iPSC mutant showed that iPSC-MNs are more vulnerable to oxidative stress, or after modulation of PI3Kt-AKT pathway, which is implicated in apoptosis (Bilican et al. 2012; W. Wang et al. 2013). In mutant C9orf72 iPSC-MNs decreased survival was reported by Imamura and collaborators, however their MNs cultures contained only 62% of HB9+ MNs thus the remaining glia cells could have contributed to the vulnerability of MNs (Imamura et al. 2017). FUS mutants iPSC derived MNs demonstrated distal axonal degradation and MNs death only tardily in the culture (100 days) (Naumann et al. 2018). Similarly, to the observations in SOD1, TDP-43 and C9orf72 iPSC-MNs, mutant FUS iPSC-MNs was most vulnerable under stress conditions compared to basal conditions (Ichiyanagi et al. 2016; Naujock et al. 2016).

Proteins aggregates in iPSC derived familial ALS MNs

The shared pathological hallmark of most ALS cases is the TDP-43 positive ubiquitinated inclusions, paralleled by mislocalisation of TDP-43, which accumulate predominantly in the cytoplasm (Arai et al. 2006; M. Neumann et al. 2006). This pathological hallmark is not observed in all ALS iPSC derived MNs. This may be due to culture conditions or the fact that a second stressor is needed to unravel less aggressive mutation phenotypes (Hawrot, Imhof, and Wainger 2020; Bilican et al. 2012; Egawa et al. 2012; Fujimori et al. 2018; Osaki, Uzel, and Kamm 2018). Several studies have reported NFs aggregates in iPSC derived ALS MNs (H. Chen et al. 2014) similarly to observations in ALS patients' post mortem tissues.

Regarding mutation specific aggregates, studies have not unanimously identified SOD1 aggregates in iPSC derived SOD1 MNs from different cell lines. Under basal conditions several studies did not observe these aggregates, while some others report accumulation of misfolded SOD1 (Kiskinis et al. 2014; H. Chen et al. 2014; Imamura et al. 2017). This may be due to low levels of SOD1 in human ALS neurons compared to transgenic animal models. Indeed, transgenic animal models with low levels of mutant SOD1 do not show SOD1 aggregates neither (Gurney et al. 1994). In contrast to SOD1 and TDP-43 iPSC derived MNs, pathogenic FUS mislocalisation to the cytoplasm and accumulation in stress granules have been uniformly confirmed by several studies in FUS mutant iPSC derived MNs (W. Guo et al. 2017; Higelin et al. 2016; Ichiyanagi et al. 2016; Lenzi et al. 2015; Marrone et al. 2018; Naumann et al. 2018).

Most studies in iPSC derived c9orf72 mutated MNs confirmed the expected neuropathological disease features. Intranuclear and cytoplasmic RNA foci were also observed in C9orf72 mutated iPSC MNs (Almeida et al. 2013; Donnelly et al. 2013; Sareen et al. 2013). Poly(GP) was also identified in a subset of C9orf72 mutated iPSC MNs (Almeida et al. 2013; Donnelly et al. 2013), though some studies reported no RAN translation in iPSC MNs from C9orf72 patients (Gomez-Deza et al. 2015).

Overall, in ALS iPSC-MNs although some mild pathological phenotypes are observed under basal conditions most of the ALS phenotypes are observed in vitro only after addition of specific cellular and DNA damage stressors. This holds true not only for the presence of aggregates but also for

neuronal survival in culture suggesting that neuronal environment may contribute to MNs degeneration.

Cellular pathways perturbed in ALS iPSC-MNs

Besides these pathological hallmarks, numerous other cellular disturbances were reported in ALS iPSC-MNs, which were also observed in animal studies. Some defects are shared between different ALS mutated iPSC-MNs, such as axonal transport defects (Egawa et al. 2012; Kiskinis et al. 2014; Alami et al. 2014; Kreiter et al. 2018; W. Guo et al. 2017; Naumann et al. 2018) perturbed neuronal excitability (A. Zhao, Pan, and Cai 2020; Devlin et al. 2015; Wainger et al. 2014; Sareen et al. 2013; Naujock et al. 2016), mitochondrial and ER stress (Bhinge et al. 2017, 201; Kiskinis et al. 2014; P. Wang et al. 2019; Wainger et al. 2014; Dafinca et al. 2016) or defects in proteostasis (Almeida et al. 2013; Farg et al. 2014; Gomez-Deza et al. 2015; Shi et al. 2018; Barmada et al. 2014; Marrone et al. 2018).

While other defects are more specific to some ALS mutations such as stress granule formation in TDP-43 and FUS mutants although it was also observed in C9orf72 iPSC-MNs (Lenzi et al. 2015; Marrone et al. 2018; Dafinca et al. 2016), nucleo-cytoplasmic transport has been mostly described in TDP-43 and C9orf72 mutants (C.-C. Chou et al. 2018; K. Zhang et al. 2015; S. Moore et al. 2019), abnormal miRNA expression mostly linked to FUS and TDP-43 mutants (Santis et al. 2017; H. Chen et al. 2014) and sensitivity to glutamate stress more predominantly in C9orf72 MNs (Donnelly et al. 2013; Selvaraj et al. 2018; Shi et al. 2018),

iPSC derived MNs have also been a valuable tool for drug screening. Perturbation of MNs excitability being a shared hallmark between a number of ALS iPSC-MNs the anti-epileptic drug, retigabine has been investigated in SOD1 and C9orf72 mutated iPSC-MNs. Interestingly, it demonstrated positive effects by reducing neuronal excitability and improved cell survival (Wainger et al. 2014). The positive results obtained in iPSC elicited a double blind phase II randomised clinical trial with 65 enrolled patients (NCT# 02450552). This clinical trial ended in 2019 and proved exciting results (Wainger et al. 2021). In fact, a dose dependent decrease in cortical and spinal MNs excitability was observed in ALS patients in comparison to controls (Wainger et al. 2021). Bosutinib, a Src/c-Abl inhibitor able to inhibit cell death and avoid the aggregation of misfolded SOD1 inducing autophagy, has also been translated into a clinical trial (UMIN000036295) (Imamura et al. 2017; Okano et al. 2020).

Modelling sALS with iPSC

In sALS patients, the most prominent pathological feature that has been described is the presence of TDP-43. Four different studies have generated sporadic iPSC-MNs (Alves et al. 2015; Burkhardt et al. 2013; Fujimori et al. 2018; Imamura et al. 2017). TDP-43 cytosolic aggregation was observed in most of the sporadic iPSC-MNs in Fujimori and collaborators study, while Burkhardt et al. reported only scarce presence of TDP-43 aggregation in their sporadic iPSC-MNs (Burkhardt et al. 2013; Imamura et al. 2017). In the study conducted by Fujimori and collaborators, several sporadic iPSC cell lines were compared and interestingly they found that the sporadic iPSC cell lines behave differently with some of them displaying FUS, TDP-43 or SOD1 aggregates. Interestingly, they showed that sporadic iPSC-MNs could be classified based on their in vitro characteristics. Moreover, the in vitro classification correlated also with some in vivo clinical phenotypes. Several other pathological phenotypes were reported in sporadic iPSC-MNs such as decreased neuronal survival, or mitochondrial defects (Alves et al. 2015; Fujimori et al. 2018).

Due to the important differences in epigenetic landscape between sALS patients, it is useful to include a high number of cases to enable the generation of informative statistical analysis. A broad study which included 32 sALS patients showed that the in vitro phenotypes of the MNs were correlated with clinical disease courses (Fujimori et al. 2018). Further, they used these cells to test potential therapeutics and could identify a subset of sALS patients which were responsive for the drug suggesting that some disease mechanisms may be shared between different patients (UMIN000034954; Okano et al. 2020). This shows the importance of including a high number of patients in order to be able to subdivide these cases based on their genome-derived characteristics and possibly identify common pathological mechanisms between different groups of sALS patients.

Taken together, the non-exhaustive list of observed pathological hallmarks in iPSC derived ALS MNs shows that iPSC MNs do recapitulate some important phenotypes observed in patients or in animal models. Additionally, interestingly, in vitro iPSC MNs phenotypes also correlate with patients' clinical features, and make it possible to identify patients that are more responsive to certain therapeutics versus others. Some clinical trials are ongoing, and some have already proven encouraging results. This supports the use of this model to study specific disease phenotypes in

complement to other more integrated studies. Nonetheless, it is important to keep in mind that since no stringent criteria exist regarding iPSC MNs differentiation, it is sometimes difficult to compare results from different laboratories which used distinct differentiation protocols. Moreover, it seems that the culture condition as well as second stressor may be required in order to make apparent some of the pathological phenotypes suggesting that in vitro mono-cultures of MNs do not recapitulate the in vitro environment. With the emergence of co-cultures one may acquire better insight in the non cell autonomous importance in different ALS subtypes.

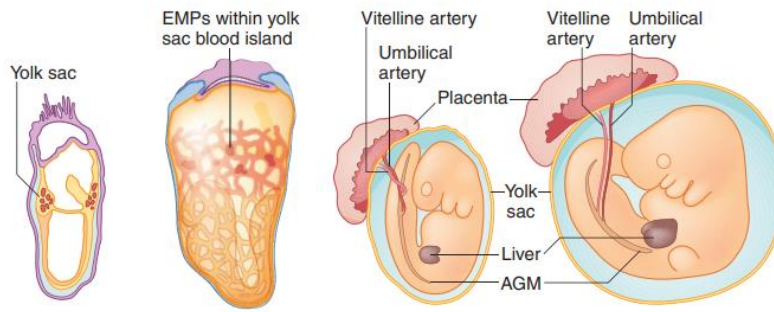
6.2 iPSC derived innate immune cells to study neuroinflammation

The majority of the protocols which to date allow the generation of immune cells have been based on the understanding of embryonic haematopoiesis. Here we will focus on macrophages and microglial cells. Although microglial cells and macrophages are similar in many ways, they remain two distinct cell types. Therefore, it is important to understand their ontogeny in order to better identify what are their commonalities but also the factors that govern their unique identity, which will help to better orientate in vitro generation and maturation of these cells in order to mimic as closely as possible the in vivo cell types.

6.2.1 Innate immune cell ontogenies

Overview of haematopoiesis

Macrophages and microglia emerge from fetal haematopoiesis, which is a dynamic and highly regulated process extending from embryonic to adult life. Most of our understanding of haematopoiesis comes from studies in rodent models. In the latter, haematopoiesis is constituted of three waves partially overlapping in time and tissues making the understanding of different tissue resident macrophages ontogeny difficult to appreciate (Figure 18). The figure 18 recapitulate the different waves of haematopoiesis, which are emerging in different regions and give rise to distinct progenitors.



| Embryonic stage | | | | |
|-----------------|--|--|--|---|
| | E7 | E8.25 | E9 | E10.5 |
| Wave 1 | Primitive erythroid progenitors macrophages megakaryocytes | Primitive erythroid progenitors macrophages megakaryocytes | Primitive macrophages | Primitive macrophages |
| Wave 2 | | EMPs | EMPs B lymphoid cells T lymphoid cells | EMPs (yolk sac) B lymphoid cells (AGM, yolk sac) T lymphoid cells (AGM, yolk sac) |
| Wave 3 | | | | Hematopoietic stem cells (AGM) |

Figure 18 Schematic representation of the different foetal hematopoietic waves during mouse embryonic development. Three different distinct waves occur which are partially overlapping and generate different progenitors; The first wave termed primitive haematopoiesis arises in the posterior plate mesoderm in the blood island from E7 and gives rise to primitive progenitors from E7.25. The second wave, termed transient definitive wave, arises in the hemogenic endothelium in the YS from E8.0 and gives rise to erythomyeloid precursors (EMPs). As soon as the blood

circulation is established EMPS migrate into the fetal liver (FL), where they subsequently expand and differentiate into different lineage cells. Lastly, almost overlapping with the second wave, the third wave termed definitive haematopoiesis emerges at E8.5 in the hemogenic endothelium. This third wave first gives rise to immature hematopoietic stem cells (HSC) in the para-aortic splanchnopleura (P-Sp) region and subsequently gives rise to foetal HSC at E10.5 in the aorta, gonads and mesonephros (AGM) regions. These progenitors further migrate in the FL where they establish definitive haematopoiesis. They also seed the fetal BM, which eventually contribute to adult HSC in the BM. Yoder et al., 2014

Tissue resident Macrophages ontogeny

Although macrophage's embryonic ontogeny is now recognised, several questions remain such as which precise progenitors contribute to foetal MP populations, what are the transcription factors that govern their identity as well as what are the differentiation routes followed by the progenitors for the emergence of distinct MP populations. In the past decade, fate mapping analysis, using lineage tracing experiments during mice embryonic life have allowed a better understanding of myeloid cells ontogeny (F. Ginhoux et al. 2010; Kierdorf et al. 2013; Gomez Perdiguero, Schulz, and Geissmann 2013; Gomez Perdiguero et al. 2015; Schulz et al. 2012; Jianpeng Sheng, Ruedl, and Karjalainen 2015). Three main theories have emerged regarding the macrophages ontogeny. These different models differ on timing and fate of the progenitors.

According to the first model most of the tissue resident macrophages are from the transient wave (second wave) erythromyeloid progenitors (EMP) from the Yolk-sac (YS) hemogenic endothelium, most of these progenitors directly colonise brain and tissues from E9 onward without transitioning through a monocytic intermediate. EMPs are transitioning to a pre-macrophages (preMac) stage,

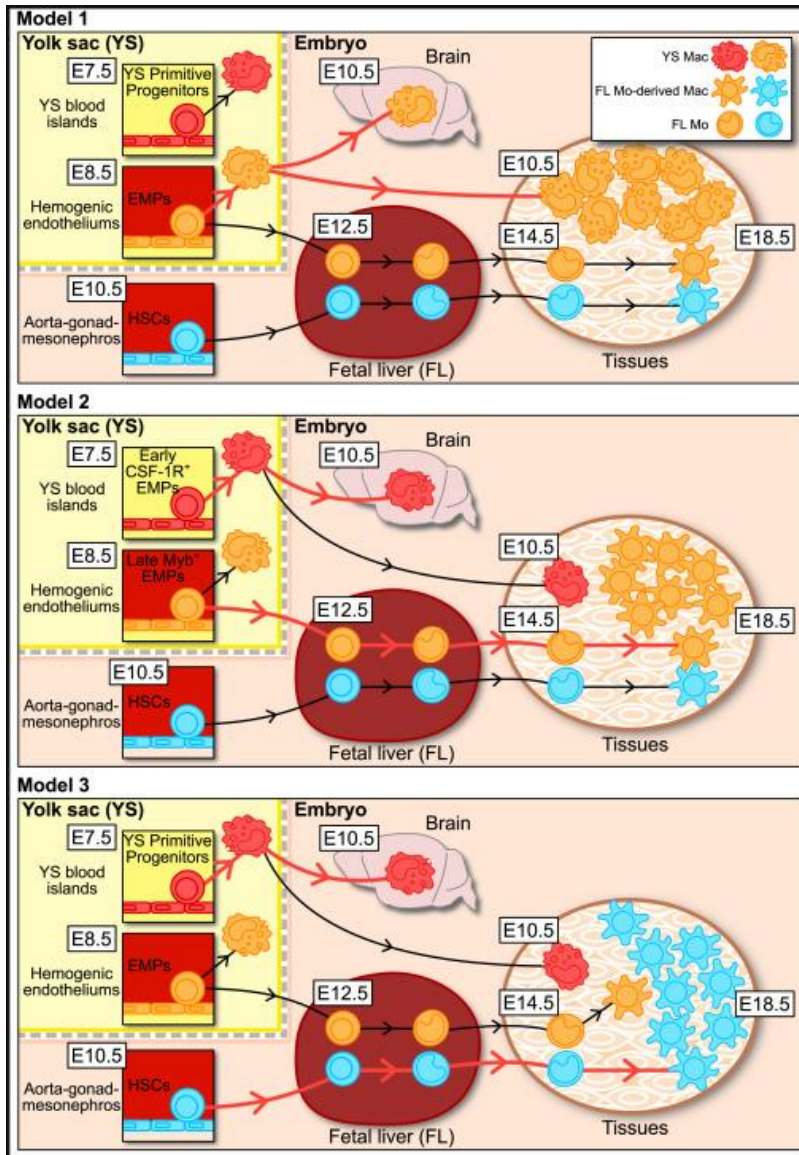


Figure 19 The different models of fetal macrophages ontogeny. Model 1 correspond to the model proposed by Gomez-Perdiguero et al., 2015. Model 2 correspond to the work of Hoeffel et al., and the third model to the work of Sheng et al., 2015

which is CX3CR1⁺ distinct from monocytes (Mass et al. 2016). EMP and preMac are expanding in the YS and enter the bloodstream from E10.5 onward to migrate to the embryo (Stremmel et al. 2018). From E9-10.5 part of the EMP population invades Foetal liver (FL) where they acquire their full potential which will give rise to erythroid and myeloid cells populations until E16.5 (Gomez Perdiguero et al. 2015). Part of myeloid progenitors will differentiate into monocytes. The latter population only colonises a small fraction of the tissues, barely diluting the initial YS macrophages population, which will represent the majority of postnatal tissue resident macrophages.

In the second model the main source of macrophages at birth are also thought to deriving from late EMPs of the transient wave, which are emerging at E8.5 in YS haemogenic endothelium. These CSFR- MYB⁺ progenitors will for

the majority migrate into the FL from E9.5 onward, where they will upregulate CSFR and become myeloid progenitors (Hoeffel et al. 2015; Florent Ginhoux et al. 2016). Amongst them are derived FL monocytes which are highly proliferative in E12.5 embryos. At E14.5 FL monocytes start invading all tissues except the brain where BBB started to form (E13.5) (Kierdorf et al. 2013; Gomez Perdiguero et al. 2015) and become FL derived tissue macrophages diluting the original YS macrophages population which was derived from the primitive wave (E7.5) of haematopoiesis. The third model resembles the second model. However, they proposed that the major contributors of adult tissue resident macrophages are derived from foetal hematopoietic stem cells (HSC) (Jianpeng Sheng, Ruedl, and Karjalainen 2015).

In conclusion, during embryonic haematopoiesis, the first model supports, a direct colonisation of EMP transitioning to preMac precursors distinct from monocytes and colonising most of the tissues; while the second theory suggests that most of the tissue resident macrophages are derived from late EMP which transit through a monocytic intermediate in the foetal liver. In the third model, tissue-resident macrophages are thought to derive from HSC, which arise with late haematopoiesis. The discordances are mainly due to the use of different promoters of inducible models and absence are reliable markers for early progenitors. Although the different studies agree to support a partial replacement of tissue resident macrophages through definitive haematopoiesis, an HSC origin for tissue resident macrophages remains controversial. In fact, a new paradigm suggests that most tissue resident macrophages self-maintain independently of bone marrow derived cells, driven by a core macrophage transcriptional program and specific tissue transcriptional regulators (Mass et al. 2016). However, why certain populations and not others are subject to this capability remain unclear. Thus, at least in postnatal mice, most organs contain a mixture of tissue resident macrophages seeded during embryogenesis and which can be maintained by local self-renewal, and a population of HSC derived macrophages originated from the circulation.

Microglial cells ontogeny: a unique subtype of tissue resident macrophage

Albeit persisting discordances on the respective contribution of yolk sac macrophages and foetal liver monocytes to final peripheral tissue-resident macrophages populations, a consensus view holds that microglia originate from YS EMP derived macrophages without transitioning by

monocytic precursor cell (Figure 19, F. Ginhoux et al. 2010; Kierdorf et al. 2013; Schulz et al. 2012). Early studies in mice models observe microglial cells in the parenchyma as early as E8 thus before the second hematopoietic wave takes place, suggesting that microglial cells originate at least partially from the first wave of haematopoiesis (Alliot, Godin, and Pessac 1999). Fate mapping studies confirmed these observations and identified primitive CD45⁻ c-kit⁺ EMP cells to be precursors of microglial cells. These progenitors mature in a RUNX Family Transcription Factor 1 (RUNX1), Spi-1 Proto-Oncogene (PU.1) and Interferon Regulatory Factor 8 (Irf8) dependent manner, and conversely to monocyte-derived macrophages, do not require the transcription factor Myb for their development (Kierdorf et al. 2013; Schulz et al. 2012). Subsequently these CD45⁻ c-kit⁺ EMPs develop transitioning between two different states: in the immature state, they upregulate CD45 and downregulate C-kit expression, while being CX3CR1 negative (A1 immature progenitors), they then mature into CD45⁺, C-kit⁻ and CX3CR1⁺ cells (A2). From E9.5 onward, concomitant to the establishment of the primary blood circulation system, the primitive macrophages migrate towards the neural tube and populate the developing brain and spinal cord where they proliferate and give rise to microglia. Importantly, it seems that the environment constitutes an essential component in the maturation and specialisation of the microglial cells. In fact, interleukin 34 (IL-34; a ligand of CSF1R) expression by neuronal cells, and secretion is essential during this migration step, which is sensed by A1 and A2 progenitors expressing CSF1R (F. Ginhoux et al. 2010; Y. Wang et al. 2012). Additionally, CSF1R signalling is essential to mediate the survival of adult microglia (Elmore et al. 2014). Interestingly, it seems that microglia developmental processes are independent of major chemokines, but require specific matrix metalloproteinases (MMP) action for tissue remodelling (Kierdorf et al. 2013).

At around E13.5 the establishment of the blood-brain barrier (BBB) prevents further colonisation of the brain by other hematopoietic progenitors. During the late period of gestation and early postnatal life, the microglial population proliferates locally and distributes spatially in the CNS. The microglial population is then maintained in the adult CNS by a constant balance between proliferation and apoptosis (Askew et al. 2017; Sharaf, Krieglstein, and Spittau 2013). Under physiological conditions, microglial cells are able to self-renew independently of peripheral progenitors, and there is no recruitment of peripheral monocytes from peripheral blood circulation in the brain (Ajami et al. 2011; Bruttger et al. 2015; Gomez Perdiguero, Schulz, and Geissmann 2013).

The current knowledge regarding microglia development is primarily based on experimental animal studies with sparse contribution from human studies of aborted human embryos, which are difficult to obtain. Recent single cell transcriptomic studies of early human embryos aborted have shed light on specific developmental progenitors in an unbiased and unsupervised manner (Z. Bian et al. 2020; Kracht et al. 2020). Although these studies still hold limitations regarding the identification of the fate of specific progenitors and their contribution in tissue resident macrophages populations, they have permitted a parallel between observations made in rodent models. Bian and collaborators have identified two HSC independent waves of haematopoiesis corresponding to what has been observed in rodent models (Z. Bian et al. 2020). Most importantly, these studies bring new tools to understand the ontogeny of microglia cells in a human context and offer new bases to mimic embryonic stages for the generation of iPSC derived microglia cells.

6.2.2 iPSC derived innate immune cells

The studies conducted in mice have allowed better understanding of haematopoiesis main steps and characterised more specifically specific pathways progenitors. These studies have been valuable for the in vitro generation of immune cells, which have based their protocol development on the knowledge acquired from these studies.

Generation of hematopoietic progenitors and differentiation into macrophages-like cells

The first protocols that aimed to generate immune cells in vitro focused on the generation of mononuclear phagocytes including macrophages and dendritic cells from mouse and human pluripotent Embryonic stem cells (ESC) (Cai et al. 2007; Lindmark et al. 2004; Fairchild et al. 2000). Although these protocols allowed generation of cells that expressed specific macrophages or dendritic markers, what rendered them complicated to use is the fact that they require co-cultures with cell lines, complex cytokines cocktails, and purification steps. From these limitations emerged new protocols using murine and human ESC and iPSC, able to yield an homogeneous population of CD14+ monocytes without requiring additional purification, and the use of complex cytokine cocktails (Karlsson et al. 2008; Senju et al. 2009; Wilgenburg et al. 2013; Yanagimachi et al. 2013). These monocytes-like cells had distinct myeloid signatures and were able to differentiate into functional macrophages.

In vitro Gastrulation

In order to have proper cell differentiation, it has been shown that In vitro cell must pass through an aggregation step termed Embryoid bodies (EBs), which mimics gastrulation. These EBs expressed genes involved in the three embryonic germ layers (Itskovitz-Eldor et al. 2000; Höpfl, Gassmann, and Desbaillets 2004). Forced aggregation of ESCs into EBs using centrifugation has shown to be efficient to initiate a better and more robust directed differentiation of ESCs (E. S. Ng et al. 2005). Subsequently, different factors are added to orientate the development in order to recapitulate hematopoietic stages.

Essential factors for early haematopoiesis in vitro

Several factors are shown essential in vitro to induce generation of hematopoietic progenitors. They are listed herein:

- Bone morphogenetic protein 4 (BMP4), is a member of the transforming growth factor – β superfamily of secreted signalling molecules. During development, BMP4 regulates mesodermal cell commitment to the hematopoietic lineage. In vitro, BMP4 addition was shown to strongly promote hematopoietic differentiation and was sufficient to induce expression of primitive streak genes and genes marking hematopoietic mesoderm (Chadwick et al. 2003; R. N. Wang et al. 2014).
- Vascular endothelial growth factor (VEGF) is a secreted growth factor. VEGF/receptor signalling is involved in the regulation of angiogenesis and haematopoiesis (H.-P. Gerber and Ferrara 2003). In vitro, VEGF addition was required for efficient generation of hematopoietic progenitor cells (Pick et al. 2007).
- Fibroblast growth factor 2 (FGF2) is an angiogenic and pleiotropic growth factor involved in the proliferation and differentiation of several cell types. FGF2 is thought to play an important role in the mesoderm induction. FGF2 positively regulates haematopoiesis by acting on stromal cells, and on early and committed hematopoietic progenitors as well as possibly on some mature blood cells. In vitro, early addition of FGF2 in the culture media was shown to increase cell yield during human ESC differentiation(Pick et al. 2007; Allouche and Bikfalvi 1995).

- Stem cell factor (SCF) also known as kit ligand, is a hematopoietic cytokine that triggers its effect by binding to the receptor c-kit expressed on early hematopoietic progenitors. Several studies suggested that SCF could promote survival of hematopoietic stem cells in vitro. In vivo it has been suggested that SCF promotes stem cell self-renewal. In vitro the adjunction of SCF with other cytokines yield a 20 fold increase in number of granulocytes, erythroids, macrophages and megakaryocytes (C. Li and Johnson 1994; Bernstein, Andrews, and Zsebo 1991; Migliaccio et al. 1992; Broudy 1997).
- Thrombopoietin (TPO) is a hormone, which was shown to be the main regulator of HSC and progenitors self-renewal and survival in vivo and in vitro. It binds to the receptor c-MPL to activate cell signalling via JAK-STAT and other pathways (Fox et al. 2002; Alvarado et al. 2017; Decker et al. 2018).
- Flt-3 ligand (Flt3L) is a member of a small family of cytokines. In vivo, Flt3L has been shown to be highly involved in the regulation of early haematopoiesis, by stimulating the development of primitive multipotent precursors and myeloid precursors (McKenna and Lyman 1996). In vitro, Flt3L acts as a tyrosine kinase receptor ligand that stimulate proliferation of hematopoietic progenitors. A study showed that hematopoietic development is improved by addition of SCF, TPO and Flt3L in vitro (Tian et al. 2004). However, these factors are not sufficient by themselves to promote differentiation into hematopoietic progenitors.
- Interleukin-3 (IL-3) is a hematopoietic cytokine which was shown to stimulate multipotential hematopoietic progenitors as well as lineage committed cells (Hara and Miyajima 1996). Interestingly, we have shown in the chapter “macrophages ontogeny” the importance of Runx1 for proper hematopoietic development. Interleukin-3 is a Runx1 target, and it was shown that in Runx1 haploinsufficient mice, IL-3 amplifies HSC from E11 AGM, YS and placenta (Robin et al. 2006). IL-3 seems to function as a proliferation and survival factor for HSC. Most of the protocols thus also add IL-3 during the developmental

stages to recapitulate embryogenesis. In addition, IL-3 stimulates proliferation of all myeloid lineage cells when supplemented with other cytokines such as granulocyte-macrophages colony stimulating factor (GM-CSF).

To date the different protocols which generate iPSC derived CD34+ hematopoietic progenitors use a combination of these different cocktails of factors in a timing dependent manner in order to recapitulate the hematopoietic stages (Yanagimachi et al. 2013; Wilgenburg et al. 2013; Lachmann et al. 2015). Emergence of CD34+ hematopoietic progenitors were observed at day 12 of the culture and diminished with time although they were still present at day 33, while CD14+ CD45+ cells increased with time (Wilgenburg et al. 2013). From day, 14 onwards myeloid cells progenitors could be collected in the supernatant. These cells can be differentiated in macrophages, dendritic cells or granulocytes depending on the protocols (Yanagimachi et al. 2013; Wilgenburg et al. 2013; Lachmann et al. 2015). These progenitors can be continuously harvested for several months, while keeping the ability to differentiate into macrophages (Yanagimachi et al. 2013; Wilgenburg et al. 2013). Very recently a protocol have been published which was adapted on the protocol from Wilgenburg et al. This new protocol claimed to allow generation of progenitors with an increase of 5-7 fold the initial yield. Interestingly, they proposed a way of overcoming the conservation limitations of these progenitors by prolonged cultivation of the latter in suspension in so called “spinner” cultures. These cultures can be maintained up to 8 weeks, and cell viability scores were above 95% after this time. The authors did not observed differences in expression markers or functional capacities when they differentiated these progenitors into macrophages compared to freshly harvester progenitors (Gutbier et al. 2020). This protocol offers new tools to yield a high number of human macrophages, which could serve for drug screening.

Differentiation into macrophages

These myeloid progenitor cells can subsequently be differentiated in macrophages via exposition to M-CSF or GM-CSF during 3-7 days (Yanagimachi et al. 2013; Wilgenburg et al. 2013; Xue et al. 2014). M-CSF and GM-CSF are main driving forces for the generation but more precisely for the terminal differentiation of functional cells of the granulocytic or macrophages lineages (Sengupta et al. 1988; Welte et al. 1985). In vitro use of GM-CSF to promote macrophages differentiation give rise to a more pro-inflammatory phenotype, while M-CSF exposure result in a more anti-

inflammatory phenotype (Sica and Mantovani 2012; Xue et al. 2014). These macrophages demonstrated typical characteristics including: (i) the capacity to phagocytosis particles, endocytosed lipoprotein and can be infected by HIV virus, (ii) the ability to secrete cytokines in response to LPS stimulation and be activated differentially with different set of cytokines (Yanagimachi et al. 2013; Wilgenburg et al. 2013). In vitro macrophages were shown to be able to be activated into a large spectrum of states which refine the initial appellation of M1 and M2 polarisation states (Xue et al. 2014). These different modes of activation are less binary and represent more the in vivo activation states. Interestingly, the better understanding of the spectrum of human macrophages activation possibilities represents an important step forward in understanding how these cells integrate and compute signals from their microenvironment and thus could be used to better study macrophage activation in the context of human disease.

Taken together, robust protocols to generate a high number of iPSC-derived macrophages were developed and can be used to study intrinsic defects linked to the different ALS genes, but also study their role towards MNs survival. There are still debates regarding how macrophages should be “physiologically” activated. However the extensive study from Xue et al., offer new possibilities and extend our understanding on the possible macrophages polarisation in vitro.

iPSC derived microglia-like cells

Protocols to generate iPSC-derived microglia-like cells are rather recent. As I have already described above, microglia cells' developmental origin differs from other myeloid cells. While blood monocytes derived from myeloblastosis proto-oncogene (MYB), microglia originate from MYB-independent YS preMac progenitors which colonise the brain until closure of the BBB (Gomez Perdiguero et al. 2015; Schulz et al. 2012).

Several protocols to generate iPSC-derived microglia emerged at around the same period of time in 2016-2017, their specificities are presented in the Table 10. The different protocols share more or less the same strategy based on mimicking in vivo developmental stages of microglia by exposing iPSC to different cocktails of cytokines and growth factors, modulating oxygen levels, and in some cases selecting specific progenitors using Fluorescence activated cell sorting (FACS) or Magnetic activated cell sorting (MACS).

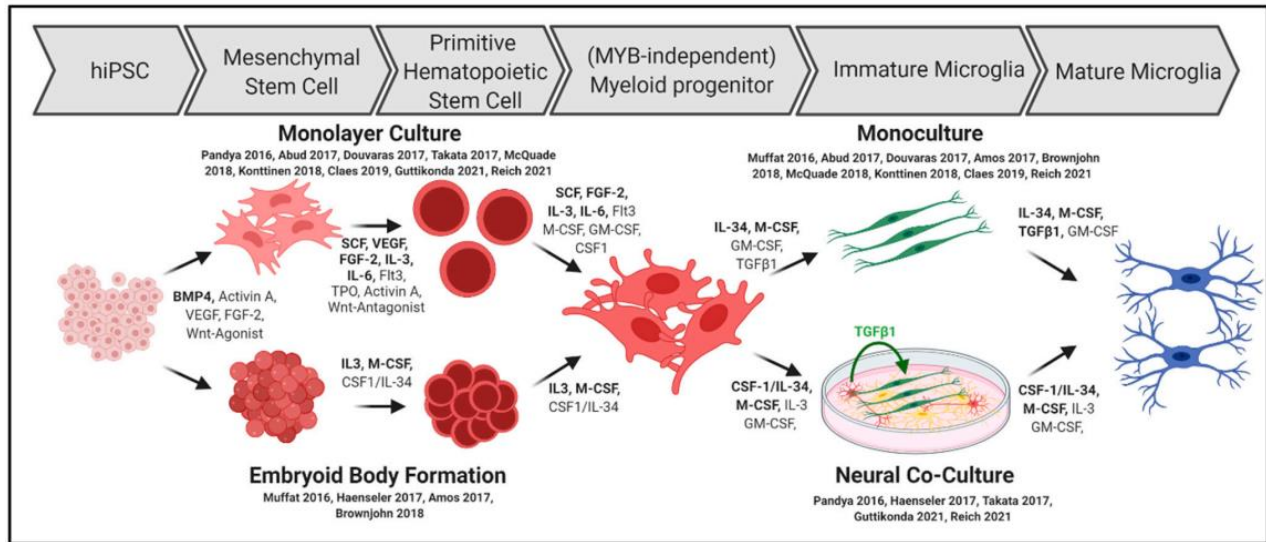


Figure 20 Recapitulation of the differentiation steps in the different iPSC derived microglia protocols. Several strategies are employed such as culture in monolayer or embryonic bodies; selection of progenitors by cell sorting; mono-culture or co-culture to help for microglial cells maturation. Wurm et al., 2021

The differentiation steps are recapitulated in the Figure 20. The most common denominator between the different protocols is the use of BMP4 to induce mesodermal commitment and mesenchymal stem cell production, as well as the use of the CSFR1 ligands, IL-34 and Colony Stimulating Factor 1 (M-CSF), for microglial cells maturation.

Generation of microglia progenitors

Regarding the first differentiation step some of the protocols are using 2D cultures (Pandya et al. 2017; Douvaras et al. 2017; Takata et al. 2017; McQuade et al. 2018; Claes et al. 2019; Konttinen et al. 2019; Reich et al. 2021), while other transit through EBs stages (Muffat et al. 2016; Haenseler et al. 2017; Brownjohn et al. 2018; Garcia-Reitboeck et al. 2018). In the protocols using the initial step of 2D culture, most of the protocols are using low oxygen during the early period of culture. In fact, it was shown that timely controlled low oxygen condition can favour iPSC cells differentiation (Podkalicka et al. 2020; Ayabe et al. 2018). The use of EBs is argued to also recapitulate this low oxygen environment (Winkle, Gates, and Kallos 2012). Additionally, the culture in EBs also confer a more physiological way of culture by formation of three germ layer structures that partially recapitulate early embryonic development (Kurosawa 2007).

The activation and inhibition of wnt-signalling pathway in a strict timely dependent manner has been shown to promote generation of either primitive haematopoietic hemangioblast precursors (KDR+CD235+) or definite hematopoietic precursors (KDR+CD235A-) (Sturgeon et al. 2014). The activation of WNT signalling is induced by CHIR99021, and promote the differentiation of iPSC into primitive streak mesodermal cells. Subsequently inhibition of WNT signalling using Activin A promote the final generation of these hematopoietic hemangioblast precursors (Sturgeon et al. 2014; Guttikonda et al. 2021). Some of the proposed protocols are using WNT activator and inhibitor to generate early haematopoietic hemangioblast precursors (Takata et al. 2017; Konttinen et al. 2019; Guttikonda et al. 2021; Abud et al. 2017). In the other studies, the induction of haematopoiesis use BMP4, VEGF and SCF giving rise to CD43+, which are hematopoietic progenitor cells (Sidney et al. 2014; Haenseler et al. 2017; Douvaras et al. 2017; Brownjohn et al. 2018; Garcia-Reitboeck et al. 2018; Claes et al. 2019; Reich et al. 2021) . In addition, interestingly, the protocols previously published by Wilgenburg 2013 and Karlsson 2008, demonstrated that the use of these early factors generate MYB-independent, RUNX1- and PU.1- dependent precursors which correspond to the progenitors described in mice to be those giving rise to microglia (Kierdorf et al. 2013; Wilgenburg et al. 2013; Karlsson et al. 2008; Buchrieser, James, and Moore 2017; Vanhee et al. 2015).

In a second step, a cocktail of different hematopoietic cytokines induces early myeloid progenitors. The composition may vary from one protocol to another but mostly contain Flt3L, G-CSF, IL-3, IL-6, SCF, VEGF, TPO. These cytokines are well characterised as important inducers for myeloid progenitors however the individual role of each factor in the differentiation step remains poorly understood. Subsequently, myeloid progenitors start to arise in the supernatant and can be harvested several times in certain protocols (Muffat et al. 2016; Douvaras et al. 2017; Haenseler et al. 2017; Brownjohn et al. 2018; Garcia-Reitboeck et al. 2018; McQuade et al. 2018; Claes et al. 2019; Reich et al. 2021). Depending on the protocols, progenitors are characterised with different hematopoietic markers such as CD34+, CD43+, CD41+, CD45+ (Gekas and Graf 2013; Vodyanik, Thomson, and Slukvin 2006; Y. Zhu et al. 2020; Daniel et al. 2016).

In the protocol proposed by Muffat and collaborators a specific media is used which aimed to recapitulate in vitro cerebrospinal fluid. Their protocol proposes a spontaneous differentiation of

EBs into neural spheroids or YS-EBs. Myeloid cells are then harvested from these YS-EBs and subsequently matured in specific media (Muffat et al. 2016).

Maturation into functional microglia-like cells

For a long time, the molecular and functional characteristics of homeostatic microglia had not been identified. This has constituted a barrier for the understanding of healthy microglia functioning and how they change and respond to injury. Moreover, another complication was the lack of understanding of if, and how, microglial cells were different from peripheral myeloid cells. Thanks to advances in sequencing technologies, several groups concomitantly published a first transcriptional signature for homeostatic microglia in adult mice, which differed from peripheral myeloid cells. This signature comprises a set of genes including, P2RY12, Transmembrane Protein 119 (Tmem119), sialic acid binding Ig-like lectin H (Siglec-H), G protein coupled receptor 34 (Gpr34), suppressor of cytokine signalling 3 (Socs3), β -hexosaminidase subunit β (Hexb), olfactomedin-like protein 3 (Olfml3) and Fc receptor-like 5, scavenger receptor (Fcrls) (Gautier et al. 2012; Hickman et al. 2013; Chiu et al. 2013; Butovsky et al. 2014). More recent findings, have completed this signature with new genes such as Sal-like protein 1 (Sall1), Sal-like protein 2 (sall2), P2Y purinoceptor 13 (P2Ry13), Solute carrier family 2 facilitated glucose transporter member 5 (Slc2a5), Sparc, TGF-beta receptor type-1 (TGF β R1), Transcription factor AP-1 (Jun), Transcription factor MafB (MafB), Myocyte-specific enhancer factor 2A (Mef2a) (Krasemann et al. 2017; F. C. Bennett et al. 2018; Haage et al. 2019; Lund et al. 2018; Chiot et al. 2020). Nonetheless, some genes were not found in common between the different studies including G-protein coupled receptor 84 (Gpr84), G-protein coupled receptor 56 (Gpr56), Sparc (M. L. Bennett et al. 2016; F. C. Bennett et al. 2018; Lund et al. 2018; Chiot et al. 2020). Differences between the studies can be linked to contaminant from other brain macrophages when using bulk-RNA sequencing. Some recent studies using single cell RNA sequencing revealed great differences in microglial gene expression even amongst different brain regions. In fact, only three of the canonical microglial genes were uniformly expressed by all clusters (Complement C1 subcomponent subunit A (C1qa), Fcrls, TREM2) (Hammond et al. 2019). In line with these findings, Van Hove and collaborators demonstrated that microglia and other brain macrophages had distinct transcriptomic profiles and did not express the canonical microglial signature genes (Van Hove et al. 2019). Overall, homeostatic microglia specific markers seem to be Tmem119, P2RY12 and Sall1, which have been reported highly expressed in microglial cells by almost all the recent studies, yet the level of

expression of this canonical signature may vary with aging and disease (Krasemann et al. 2017). Moreover, some of the genes listed as the canonical microglia signature are also expressed in other types of macrophages, however what differs is usually the level of expression found in the different cell types (Chiot et al. 2020).

The better characterisation of microglia specific markers have helped to better orientate cell maturation in vitro in order to recapitulate the main homeostatic signature.

In maturation step, a majority of protocols are using at least IL-34 for microglial maturation. The use of this factor is supported by in vivo studies in mice which showed IL-34 to be critical for microglia maturation and survival (Y. Wang and Colonna 2014; Easley-Neal et al. 2019). Additionally it was shown that CSF1R activation is warranted for microglia survival (Elmore et al. 2014). Thus, in the cases where no IL-34 is used, usually other protocols use M-CSF, which is also a CSF1R ligand more specific to PNS in vivo. Microglia cells matured with IL-34 and M-CSF expressed characteristic markers part of the microglial signature including MER Proto-Oncogene, Tyrosine Kinase (MERTK), Integrin Subunit Beta 5 (ITGB5), CX3CR1, TGFBR1, Allograft Inflammatory Factor 1 (IBA1), Integrin Subunit Alpha M (CD11b) and Tmem119 (Muffat et al. 2016; Abud et al. 2017; Douvaras et al. 2017; Haenseler et al. 2017).

Pandya and collaborators used culture with astrocytes to induce microglia maturation suggesting that other factors secreted by astrocytes may also be potent to induce maturation. Amongst the secreted factor by astrocytes, there is the Transforming Growth Factor Beta 1 (TGF β 1).

In rodents it has been shown that TGF β 1 regulate microglia development, differentiation, activation, homeostasis, maturation and survival (Abutbul et al. 2012; Butovsky et al. 2014; Jingyi Sheng, Chen, and Zhu 2015; Utz et al. 2020; Spittau, Dokalis, and Prinz 2020). TGF β 1 action is mediated by TGF β R1, which is highly expressed on rodent microglia in vitro and in vivo. Loss of TGF β 1 in the CNS led to loss of the microglia gene signature and a decrease in number of cells starting from postnatal day 20 (Butovsky et al. 2014). In vitro, TGF β 1 removal in the culture media was shown to down regulate the expression of P2RY12, TGF β R1, CX3CR1, GPR84 and CD33 in iPSC-microglia-like cells (Abud et al. 2017). Several protocols have used TGF β 1 to induce iPSC-microglia-like cells maturation (Abud et al. 2017; Amos et al. 2017; McQuade et al. 2018; Reich et al. 2021).

In order to mimic the in vivo neuronal environment, some protocols also supplement the maturation media with CD200 and CX3CL1 (L. Zhang et al. 2018; Abud et al. 2017; McQuade et al. 2018). CD200 and CX3CL1 are molecules found on neuronal and endothelial cells. In physiological conditions, neurons and microglia can contact together by these molecules and promote homeostatic microglia response. In fact addition of CD200 and CX3CL1 in culture increased the expression of specific genes implicated in the microglia sensome and MHC-II complex genes (Abud et al. 2017; Hickman et al. 2013). Additionally these cells displayed increased phagocytic capacities suggesting that the adjunction of these two factors may prime microglia into a more functional state.

Besides usage of specific molecules to induce microglia maturation, several protocols are using culture onto neuronal cells or astrocytes to induce microglia-like cells proper maturation (Pandya et al. 2017; Haenseler et al. 2017; Takata et al. 2017; Reich et al. 2021; Guttikonda et al. 2021). In fact, monocultures may not always recapitulate the complex signalling governing microglia maturation in vivo. Microglia cultured with neuronal cells have a distinct transcriptomic profile. They expressed human specific sialic acid-binding immunoglobulin-type lectin (SIGLEC) 11 and SIGLEC 12, which interact with neuronal glycoalyx and are involved in neuroprotection and suppression of inflammatory response (Linnartz and Neumann 2013; Abud et al. 2017). Additionally, Haenseler and collaborators showed that microglia-like cells co-cultured with neurons highly express the microglia genes including MERTK, GPR34, PROS1, C1QA, GAS6 and P2RY12. Moreover, the level of expression of these different markers was similar to directly processed adult human microglia, whereas, although in blood monocytes derived macrophages the expression of certain of these markers was also reported, the level of expression did not reach the level of microglia-like cells (Haenseler et al. 2017).

Taken together, several distinct protocols now permit generation of microglia-like cells. Transcriptional, molecular and functional studies showed that the obtained cells were microglial-like cells. It appears that several factors are essential for proper microglia development and maturation including IL-34 and TGF β 1. Additionally, owing to the complexity of the neuronal microenvironment, maturation over neuronal cells appear to bring further maturation to microglia

cells which may better recapitulate in vivo cues. To date the maturation mechanisms and crosstalk with environmental cells which govern specification of microglial phenotypes remain still poorly described. Nonetheless, studies using iPSC derived microglia-like cells recapitulate some of the pathological signatures, and thus show that these cells can be used to explore disease mechanisms in various ND (Brownjohn et al. 2018; Garcia-Reitboeck et al. 2018; Reich et al. 2021). In the context of ALS disease, the investigation of iPSC derived microglial cells have not yet been performed. However, with the description I made in the previous chapters one could see the importance of neuroinflammation in ALS pathogenesis, supporting the interest of using iPSC technologies to better understand the role of neuroinflammation in different ALS disease contexts.

Table 10 Protocols to generate iPSC derived microglia

| First Author | Initial culture technique | progenitor harvest | Expressed markers on Progenitors | # of harvest(s) | Sorting of progenitors | Maturation media Microglia | Obtention of microglia | Markers MG | Needing co-culture for maturation | Yield | Purity |
|-----------------------|---------------------------------|---------------------------|--|-----------------|-----------------------------------|--|------------------------|--|-----------------------------------|--------|--|
| Muffat et al. 2016 | Ebs Two types | Trituration of cystic Ebs | VE-cad+, ckit+, CD41+, CD235a+ | 6 | No | IL-34, M-CSF | D74 | TMEM119+, P2RY12+, Iba1+, CD45+ | No | 0.5-4x | Majority of cells TMEM119+, Iba1+, CD45+ |
| Pandya et al. 2017 | 2D cultures Day 0-10 low oxygen | In the supernatant | CD34+, CD43+, CD45+ | 1 | No | GM-CSF, M-CSF, IL-3 | D22-29 | HLA-DR+, CD45+, TREM2+ and CX3CR1+ CD86-, CD206-, CD200R- and CD80 - | Yes on astrocytes | 0.8-3x | 9% CD39+ |
| Abud et al. 2017 | 2D cultures Day 0-4 low oxygen | In the supernatant | CD41+, CD43+, CD235a+ | 1 | CD43+ | M-CSF, IL-34, TGFb1 Full maturation: CD200, CX3CL1 | D38 | CX3CR1+, P2RY12+, TREM2+, TGFBR1+, MERTK+, PROS1+, ITGB5+ | No | 30-40x | 97% P2RY12+, TREM2+ |
| Douvaras et al. 2017 | 2D cultures | In the supernatant | CD309+, CD235a+ | 4 | CD14+ CX3CR1+ | IL-34, GM-CSF | D45-65 | CD11c+, Iba1+, P2RY12+, TMEM119+ | No | 2x | 68% ± 4% CD14+ |
| Haenseler et al. 2017 | Ebs | In the supernatant | CD14+, CD16+, CD45+, CD86, CD163+, HLA-DR | ++ | No | IL-34, GM-CSF | D42-70 | Iba1+, P2RY12+, TMEM119+, MERTK+ | Yes on neurons | 10-43x | 100% CD11b+, CD45+, CD14+ |
| Takata et al. 2017 | 2D cultures Day 0-8 low oxygen | In the supernatant | CD41lowint, Ckit+, CD45low, F4/80- | 1 | CD45+ CD11b+ CD163+ CD14+ CX3CR1+ | M-CSF | D46 | Iba1+ | Yes on neurons | 10-20x | 94% Iba1+ |
| Amos et al., 2017 | Ebs | In the supernatant | CD14+ | ++ | No | IL-34, TGFb1, M-CSF, GM-CSF | D40 | CSF1R+, CX3CR1+, P2RY12+, HEXB+ | No | ? | ? |
| BrownJohn et al. 2018 | Ebs | In the supernatant | CD14+, CD16+, CD45+, CD86+, CD163+, HLA-DR | ++ | No | IL-34, GM-CSF | D20-24 | Iba1+, CD45+, TREM2+ | No | 23-52x | 95.6% ± 3.6% Iba1+, 95.0% ± 3.6% CD45+ 99.5% ± 0.4% TREM2+ |

| First Author | Initial culture technique | progenitor harvest | Expressed markers on Progenitors | # of harvest(s) | Sorting of progenitors | Maturation media | Microglia | Obtention of microglia | Markers MG | Needing co-culture for maturation | Yield | Purity |
|-------------------------------|-----------------------------------|------------------------------|----------------------------------|-----------------|------------------------|---|-----------|------------------------|----------------------------------|---|----------|---|
| Garcia-Reitboeck et al., 2018 | EBs | In the supernatant | CD45+, CD11b+ | ++ | No | M-CSF | | D23-28 | P2RY12+, TMEM119+ | No | 10-43x | 100% CD11b+, CD45+, CD14+ |
| McQuade et al., 2018 | 2D cultures | In the supernatant | CD43+ | ++ | No | IL-34, TGFβ1, M-CSF Full maturation: + CD200, CX3CL1 | | D45-60 | IBa1+, P2RY12+ | No | ? | 100% CD11b+, CD45+, CD14+ |
| Claes et al., 2019 | 2D cultures | In the supernatant | CX3CR1+, CD14+ | ++ | CD14+ | IL-34, M-CSF | | D24-38 | IBa1+, TMEM119+ | No | 5-10x | 100% TMEM119+, IBa1+ |
| Konttinen et al., 2019 | 2D cultures Day 0-2 low oxygen | Loosely attached round cells | CD235a+, CD41a+ | 1 | No | IL-34, M-CSF | | D24 | IBa1+, TREM2+, TMEM119+, P2RY12+ | No | 20x | 88% CD11b+, 99% CD45+ 91% IBa1+ |
| Reich et al., 2021 | Ebs and 2D cultures | In the supernatant | CD14+, CD11b+, CD68+ | ++ | No | IL-34, M-CSF, TGFβ1 | | D44-54 | IBa1+, P2RY12+, TREM2+ | Yes on neurons and astrocytes | 132-185x | ? co-culture: 82% CX3CR1+ mono-culture: 99% CD11b+, 85% CX3CR1+, 100% PU.1+ |
| Guttikonda et al., 2021 | 2D cultures | In the supernatant | KDR+, CD235A+ | ++ | No | IL-34, M-CSF | | D25 | IBa1+, PU.1+, CX3CR1+, CD45+ | Both: - on hPSC-derived cortical neurons - mono-culture | ? | |

OBJECTIVES

The main objective of my thesis work was to compare the respective roles of macrophages and microglial cells towards MN degeneration in different ALS forms, using the iPSC technology.

When I began, Delphine Bohl's group had already constituted a bank of iPSC clones from patients carrying different ALS mutations and iPSC from sporadic cases. Cynthia Lefebvre-Omar was in the process of finalising her thesis work studying the intrinsic defects of MNs in different ALS patients. I participated to the ongoing experiments at several levels including: cultures of iPSC, differentiation of MNs (35 days of culture), isogenic controls cell lines experiments, and paper writing. This work allowed me to be the second author of the paper by Lefebvre-Omar et al. entitled: "Neurofilament accumulations in motor neurons of patients with Amyotrophic Lateral Sclerosis impair structural axonal initial segment plasticity" (Submission planed before the end of the year). The manuscript is included in the Appendices. In this work, iPSC from patients carrying mutations in the three main genes responsible for ALS - *C9ORF72*, *SOD1* and *TARDBP* - as well as isogenic and non-related iPSC clones, were differentiated into MNs. Transcriptomic analysis showed that iPSC-derived MN cultures were pure and contained the subtypes of spinal MNs mainly affected in ALS patients. The study also showed NFs accumulations at early time points of the MN differentiation kinetic. We showed that these accumulations were often localized in the proximal axonal regions of ALS iPSC-derived MNs and of patient's MNs in post-mortem tissues. In the presence of NF accumulations, significant delocalisations of the Axonal Initial Segment (AIS) and of sodium Nav channels were measured, as well as increases of axonal calibers, showing AIS structural alterations. In parallel, ALS MNs acquired altered excitability with time in culture. Taken together, our findings revealed a novel pathway by which NFs disrupt AIS motoneuronal plasticity.

In my experiments, I used the same protocol to generate MNs and study their interactions with innate immune cells at different time points. Importantly as foundation of my work, the study from Lefebvre-Omar et al. showed that ALS MNs survived as control ones in their supportive medium supplemented with neurotrophic factors, with low progressive neurite damages appearing with time in culture.

Our work and other published studies on iPSC derived MNs comforted us that iPSC models were relevant to study ALS. Subsequently the next question asked by Delphine Bohl's group turned to the understanding of the role of environmental cells in MN degeneration, with a particular interest on the role of innate immune cells. Dr. Séverine Boillée, the head of the team, made in 2006 a pioneered study showing the toxic role of microglial cells and macrophages in mutant human SOD1 mouse models (Boillée et al. 2006). More recently, a study from the laboratory showed that macrophages and microglial cells reacted differentially to MN degeneration in similar mouse models and that replacement of peripheral macrophages by more trophic macrophages was beneficial on disease progression (Chiot et al. 2020). Whether this observation could be extend to other ALS forms and in a human context appeared as an important question. In a therapeutic perspective, this question raises several interest: firstly, it is particularly interesting to understand the respective contribution of microglial cells and macrophages in different ALS forms, as they are two subtypes of innate immune cells, which are located at the vicinity of different regions of the MNs. Indeed, microglial cells are in the CNS in contact with MNs' soma while macrophages are located in the PNS in contact with MNs' axon. The distinct microenvironments where they reside could suggest that macrophages and microglia may necessitate different types of modulations in the context of disease and within each compartment, there might be also differences according to different ALS subtype. Secondly, understanding the role of macrophages in different ALS subtypes represent an interesting question as macrophages being at the periphery they represent more accessible targets in ALS patients and could be of great interest to slowing down disease progression in all ALS patients. To my knowledge, there is still no published report studying the contribution of human macrophages and microglia towards MN degeneration in different ALS forms in a human context.

In this context, **my first objective** was to set up a protocol to generate innate immune cells from iPSC. Thanks to developmental studies of the hematopoietic system, the generation of protocols allowing the differentiation of iPSC into macrophages and microglial cells appeared feasible. While there was already published protocols to generate macrophages, only one paper for the generation of iPSC-derived microglia was published when I began my work. Since then, several

protocols have been published. I spent sometimes to develop my own protocol and I succeeded to set up a protocol allowing for the first time the generation of progenitors able to be differentiated into either macrophages or microglia when in co-cultures with neurons to mimic their in vivo environment and favour their specific maturation.

My **second objective** was to study intrinsic defects in ALS macrophages and microglia. This implicated to study specific macrophages and microglia mono-cultures (these results are not included in the present manuscript), because we observed that microglial cells necessitated co-cultures with neuronal cells to ensure their proper maturation. Thus, we subsequently drove our interest on macrophages and microglial cells intrinsic defects when they were cultured over wild-type or mutant neuronal cells. My **third objective** was to assess over time the trophic or toxic support of ALS macrophages and microglia towards ALS MN. For that purpose, I realised matched and mixed cultures between MNs, macrophages or microglial cells, carrying different ALS mutations. I focused my analysis on two main functions of phagocytes: the degradation pathways involved in the degradation of MN debris in co-cultures and the secretion of inflammatory factors by innate immune cells to resolve inflammation.

In my second and third objectives I included in my experiments: 5 control iPSC clones, 11 different ALS patients iPSC clones including 3 SOD1 mutated patients, 4 C9orf72 mutated patients, 2 TARDBP mutated patients and 2 sporadic patients (for some patients we had several iPSC clones. In total I have worked with 17 iPSC clones). I also worked with 5 different isogenic control iPSC clones. In this manuscript, only 7 different ALS mutated patients iPSC clones are included (2 SOD1 mutated patients, 4 C9orf72 mutated patients, and one TARDBP mutated patient. With the use of the different clones from a same patient, the work presented herein include 13 iPSC mutant clones).

Therapeutic trials aimed at modulating inflammation have so far shown no beneficial effects for ALS patients, while in pre-clinical studies interesting effects have been previously observed on mutant human SOD1 mouse models. This reinforces the idea that defects may not be identical between the different ALS forms and that a better understanding of the mechanisms specific to each mutation would make it possible to better orientate and classify the patients who may

respond to treatment. While the effectiveness of a treatment may depend on the ALS form, the temporality of the modulation of inflammation also appears to be a key element to investigate. Indeed, in the mutant human SOD1 mouse model, it has been shown that inflammation seems to be beneficial during the early phase of the disease with a rather anti-inflammatory environment, and that with disease progression it becomes deleterious, with a more pro-inflammatory micro environment (Hooten et al. 2015). A better understanding of this temporality in other forms of ALS could help to better target the critical time window where inflammation should be twisted toward one or the other way.

RESULTS

Main results

In this chapter, I present the results of my PhD project. My main work will be first rapidly introduced and then detailed in the scientific article intended to be submitted for publication. In a second part, I present complementary results.

Article: **Roles of macrophages and microglial cells towards motor neuron degeneration in ALS.**

INTRODUCTION

ALS is the most frequent adult-onset MN disease. ALS is inexorable worsening, leading very rapidly to muscle atrophy, paralysis and death, with a median survival of 3 to 5 years after diagnosis. It is a very heterogeneous syndrome with familial forms accounting for ~10% of patients whereas sporadic ALS occurs in most cases. The major genetic causes of dominant inheritance are intronic hexanucleotide expansions in the C9ORF72 gene, and mutations in genes encoding superoxide dismutase 1 (SOD1), TAR DNA-binding protein 43 (TARDBP), and fused in sarcoma (FUS). Today few mouse models recapitulate the ALS human pathology and several mechanisms were proposed to explain MN degeneration. However, the heterogeneity of the disease as well as the absence of relevant models for all ALS forms render the understanding pathogenesis and developing therapies challenging.

In this context, the generation of unique human cellular models through the production of iPSC of patients opens new perspectives for a better understanding of the pathological mechanisms involved in the pathology.

Although MNs are the primary targets in ALS, neighboring cell types may participate, involving non-cell autonomous mechanisms. The best evidence for a contribution of mutant/diseased macrophages/microglia comes clearly from the studies of ALS mice. Reports show that reducing mutant SOD1 expression selectively in macrophages/microglia strongly slowed down disease progression in ALS mice, suggesting a contribution of diseased macrophages/microglia towards MN degeneration in ALS mice. Importantly, both macrophagic cell-types, microglia around MN cell bodies in the spinal cord and peripheral macrophages infiltrating affected motor nerves are known to react to the ongoing MN degeneration in ALS mice. Until recently, both cell types could not be targeted separately in vivo as they belong to the same myeloid lineage and share common markers.

In Chiot et al., the team showed that it was possible to distinguish macrophages and microglia in vivo and importantly showed that the two cell types react differentially to MN degeneration. In this context, the objectives of our work was to study the respective roles of macrophages and microglial cells in various ALS forms.

SUMMARY OF RESULTS

Set up of a protocol to generate macrophages and microglia from iPSC.

Figures 1, 2, 3.

The objective was to be able to generate macrophages and microglia from the same progenitors. Thanks to developmental studies of the hematopoietic system and of the origins of macrophages/microglia, and to previous protocols describing the generation of iPSC-derived macrophages, we designed an original protocol allowing the production of mature and functional iPSC-derived macrophages and iPSC-derived microglial cells. Both cell types, as well as their progenitors, were fully characterised at different levels to confirm their specific identity.

The trophic support of control and ALS innate immune cells towards MNs.

Figures 4, 5, Supplementary Figures 1 and 2.

Previous results described in Lefebvre-Omar et al. showed that ALS MNs survived like control ones in vitro when they were cultured in a medium enriched with neurotrophic factors. This MN survival was reported in many studies on ALS MN phenotypes and ALS MN death was shown to be inducible by removing trophic factors or adding other stressors to the culture medium. In the present study, we had to perform co-cultures and thus we had to find media in which both MNs and myeloid cells would survive. For that, we tried different media and found that the medium for iPS-derived macrophages and a medium enriched with different cytokine and devoid of B27 as it contains corticosterone, superoxide dismutase and catalase, allowed MN survival with significant differences between ALS and control MNs.

Under these culture conditions, we compared cultures of MNs alone, in matched and mixed cultures determine the trophic supports of ALS innate immune cells towards MNs.

Alterations of the endolysosomal pathway.

Figures 6, 7 and Supplementary Figure 3.

Phagocytosis is one of the major function of innate immune cells. As we observed clearance of cell debris in co-cultures, we asked whether this function was altered in ALS macrophages and microglial cells. Evidences were also published regarding defects in the degradation pathways in ALS cells.

Matched and mixed co-cultures were established between ALS or control macrophages and control MNs. Analysis of the endolysosomal pathway was done with quantifications of LAMP1 and EEA1 positive vesicles into macrophages. We observed deregulation of the endo-lysosomal pathway in all the different mutants albeit they presented mutation specific phenotypes. Early endosomal vesicles volumes were increased in all mutants, while lysosome vesicle volumes were increased in C9orf72^{(G4C2)ⁿ}, or decreased in SOD1^{N139D} and TARDBP^{G348C} mutants.

Figures 8, 9.

Matched and mixed co-cultures were established between ALS or control microglial-like cells and control MNs. Same analysis were done as above. Nonetheless, the deregulations observed were not identical. Surprisingly in ALS microglia we observed decreased lysosomes and early endosomes vesicle's volumes compared to controls, suggesting a common defect in this pathway in all ALS mutants compared to controls.

Modifications of secretory profiles.

Figure 10 and supplementary Figures 4 and 6.

Both phagocytosis and inflammatory secretory profiles are linked in innate immune cells. To finely analyse variations in inflammatory factor secretions, 26 inflammatory factors were measured in the supernatants of matched and mixed co-cultures between ALS macrophages and control or ALS MNs. We also measured these 26 inflammatory markers in MNs cultured alone in the macrophages media and observed very little secretion or no secretion of the factors. In co-cultures conditions we noticed three major types of secretion profiles: non-secreted factors, common ones suggesting a "classical" inflammatory response of ALS and control macrophages along contact with degenerating ALS or control MNs, and the cytokines which were specific to ALS mutants. Interestingly, we observed that the secretion of these factors could be modulated when ALS macrophages were cultured over control MNs suggesting an activation from ALS MNs. In addition

we observed an evolution of the secretion across culture aging demonstrating the capacities of our iPSC derived macrophages to respond to the external environment.

Figure 11 and supplementary Figures 5 and 7

Similarly to the co-cultures of macrophages and MNs, we have measured the same 26 inflammatory factors in mixed and matched co-cultured of control or ALS microglia and MNs. In MNs cultured alone in the microglia maturation media we measured low secretion of these different inflammatory factors. In co-cultures conditions, we witnessed similarly to macrophages co-cultures three profiles of secretion although the factors falling in each category were different between the two cell types. The “classical” response was still present in microglia cells although some differences were present. Regarding the ALS specific cytokines, different profiles of secretion were found in the different ALS mutants and were different from the ones observed in macrophages.

Taken together, these results suggest that macrophages and microglial cells respond differentially to MNs degeneration.

Altered pathways found in transcriptomic analysis

Figure 12

We performed a RNA sequencing analysis of negatively cell-sorted control and ALS microglia and macrophages cultured over matched MNs. Differential profiles of genes expression were observed between the two cell types as well as between mutant cells. Interestingly, Gene Set Enrichment Analysis reveal specific pathways deregulated in the two cell types, which supported our previous cellular and molecular observations. We also observe specific deregulated pathways between the 2 type of innate immune cells.

CONCLUSIONS

Taken as a whole, this study is the first one to compare iPSC derived ALS microglia and macrophages contribution to ALS MNs degeneration in a human context and with various ALS forms. Additionally, our study are in line with previous observations from post-mortem ALS patients’ tissues as well as animal studies. We report defects in the endo-lysosomal pathway that are cell type and mutation specific. Since the endolysosomal pathway is highly linked with the

secretory system, we drew our interested in the innate immune cells secretory profiles along ALS MNs aging in culture. We identified a “classical” inflammatory response shared amongst different ALS mutations, which appeared to be cell type specific, although some factors are common to both cell types. More interestingly, we observed some shared mutant-specific secreted factors, which could be modulated when ALS macrophages or microglia were cultured over control MNs, demonstrating an importance of ALS MNs in the inflammatory response of ALS immune cells. Our study is a proof of concept demonstrating the possibility to use iPSC derived immune cells and MNs in co-cultures to recapitulate somehow the in vivo context in ALS patients. With advances and recent years developed protocols to generate a number of different immune cell types, we hope that our model will serve also to study other ALS immune cells contribution to ALS MNs degeneration. Moreover, our co-culture models could allow a better understanding of the chronology of inflammatory events specific to each ALS form with the perspective to be able to modulate inflammation at a definite time point to act beneficially on disease progression.

Authors contributions in this article

The results presented in this article were in the majority of them generated, collected and analysed by myself. I also contributed to the writing of the manuscript.

Regarding the contribution of the other authors:

- Léa Karpf have helped me with the setting of my multiplex analysis, as well as with some of my co-cultures experiments.
- Cynthia Lefebvre Omar have contributed to the teaching and coaching for the MNs generation protocol.
- Coline Jost-Mousseau have helped me with iPSC cultures.
- Justine Guégan and Emeline Cherchame are working on the bio-informatic platform in the institute and helped me with my RNA sequencing data
- Matthieu Ribon have helped me with the setting of some initial macrophages cultures and flow cytometry protocols
- David Akbar is working on the microscopy platform and helped me with the setting of my imaging protocols
- Christian Lobsiger, Stéphanie Millecamps and Séverine Boillée contributed to some data interpretation and feedback on my work across my thesis
- Delphine Bohl contributed to the conception and design, financial support, provision of study material, data interpretation and manuscript writing

Roles of macrophages and microglial cells towards motor neuron degeneration in ALS

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Abstract

Amyotrophic Lateral Sclerosis (ALS) is characterized by a progressive degeneration of motor neurons (MNs) in the spinal cord of patients and is associated with a significant immune reactivity. Evidences exist that microglia and macrophages are activated in ALS patients but their respective contributions remain unknown. In the present study, we wanted to better understand their roles in the pathology and in particular their trophic or toxic supports towards ALS MNs during degeneration. In this context, the iPSC technology offers a unique opportunity to model interactions between MNs and innate immune cells. With this objective, we compared in a similar experimental context macrophages or microglial cells derived from iPSC of patients with different ALS forms. Firstly, we were able to set up an iPSC differentiation protocol allowing the generation of macrophages or microglial-like cells with their own specific characteristics. Secondly, when co-cultured with ALS MNs, we observed that both myeloid cell types had trophic supports towards MNs and that they were able to slow down MN degeneration. Despite these trophic behaviors, we also observed progressive alterations in their endo-lysosomal pathway, suggesting that ALS macrophages and microglial cells became progressively less efficient in their degradation capacities. Thirdly, we were able to show that ALS macrophages and ALS microglia had different secretory profiles of inflammatory factors when co-cultured with MNs and that the profiles were different with time in culture. Finally, transcriptional analysis showed similarities but also differences in gene expression between ALS macrophages and ALS microglial-like cells (both cultured onto ALS MNs), showing the different roles played by these cells during the course of the disease.

Introduction

Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disease without curative treatment leading rapidly to paralysis and death of affected individuals (Mejzini et al., 2019). ALS is characterized by a progressive degeneration of motor neurons (MNs) in the cortex and the spinal cord of patients, and is associated with a significant immune reactivity. Post-mortem studies brought the earliest observations assessing the presence of inflammatory signs in ALS patients and studies on animal models have shown that inflammation was a major player in the pathology rather than being only a consequence of neuronal death (Liu et al., 2020).

A hallmark of ALS is the degeneration of the spinal MNs, which is located in two distinct microenvironments: the central nervous system (CNS), where the MN soma is surrounded by microglial cells, the macrophages of the CNS, and the peripheral nervous system (PNS) where axons can be in contact with macrophages. In post-mortem tissues of patients with familial ALS (mutated in C9orf72, SOD1, TARDBP) and with sporadic ALS, Chiot et al. (Chiot et al., 2020) showed the presence of CD68-positive, a microglial/macrophage marker, in spinal cord ventral horns, in ventral roots and in peripheral nerves of patients, with no staining in control subjects. This suggested an activation of microglial/macrophage cells in both the CNS and the PNS in all ALS forms. The pioneered studies showing a deleterious impact of these cells in ALS come from two independent reports in 2006 demonstrating that the decreased expression of human mutant SOD1 only in macrophages/microglia in ALS mouse models led to slowing down disease progression and increased survival (Beers et al. 2006b; Boillée et al. 2006b). Strikingly, Spiller et al. presented recently the neuroprotective effects of reactive microglial cells in a TDP-43 mouse model (Spiller et al., 2018), suggesting that the toxic or trophic role of phagocytes may be context-dependent. Going one-step further, in the mutant SOD1 mouse model, Chiot et al. showed that, sciatic nerve macrophages and microglia reacted very differently to neurodegeneration, suggesting that both cell types have to be taken as two separate entities with respect to responses of the innate immune system during MN degeneration. Whether microglial cells and macrophages have different involvements during the disease course in all ALS forms, remain unclear.

In order to address this question, interactions of MNs with either macrophages or microglial cells were studied with human induced pluripotent stem cells (iPSC) generated from ALS patients mutated in C9orf72, SOD1 and TARDBP genes, and from isogenic and age- and sex-matched control subjects. First, we set up a protocol allowing the differentiation of iPSC into either

macrophages or microglia-like cells from myeloid-like progenitors. Then, we compared phenotypes of ALS MNs alone or in mixed/matched co-cultures with macrophages or microglia-like cells, to assess the contribution of these cells to MN degeneration. Next, we looked whether phagocytic capacities and cytokine secretion profiles of ALS macrophages and microglial-like cells were altered in co-cultures, and finally we carried out a transcriptomic study to identify deregulated pathways that could be of interest in the perspective to modulate the immune reaction to support ALS MN survival.

Results

Generation of iPSC-derived macrophages and microglial-like cells

To model and study interactions between MNs and either macrophages or microglial-like cells, we differentiated iPSC clones from ALS patients and controls into the three cell types. **Supplementary Table 1** summarizes the different iPSC clones used in our study. iPSC clones were derived from four patients carrying expansions in the C9orf72 gene, two patients with mutations in the SOD1 gene (N139D and H44R mutations), one patient with mutations in the TARDBP gene (G348C), and age and sex-matched controls as well as isogenic control clones.

The protocol allowing the generation of iPSC-derived MNs was already described (Guo et al., 2017; Maury et al., 2015) (Lefebvre-Omar et al.*). Cultures were pure, devoid of glial cells or proliferative progenitors, and MNs were characterised as the ones primarily affected in ALS (Lefebvre-Omar et al.*). Here we have adapted protocols of the literature (Douvaras et al., 2017; Haenseler et al., 2017; Yanagimachi et al., 2013) to differentiate from the same iPSC-derived myeloid-like progenitors, both macrophages and microglia-like cells (**Figure 1a, 1b**). Briefly, iPSC cultured as floating Embryoid bodies (EBs) were orientated towards a mesodermal differentiation and the generation of primitive-streak like cells with the bone morphogenetic protein 4 (BMP4) (Richter et al., 2014). After 4 days, vascular endothelial growth factor (VEGF), stem cell factor (SCF) and basic fibroblast growth factor 2 (FGF2) were added in cultures to induce hemangioblast-like progenitor production (Abo-Rady et al., 2020). A cocktail of hematopoietic cytokines (macrophages-colony stimulating factor (M-CSF), FMS-like tyrosine kinase 3 ligand (FLT3-L), SCF, interleukin-3 (IL-3) and thrombopoietin (TPO)) was then added at day 10 to induce maturation into myeloid-like progenitors (de Graaf & Metcalf, 2011; Metcalf, 2008; Robb, 2007). Floating progenitors were harvested once every 3-4 days. Analysis by flow cytometry showed that

each harvest contained more than 98% of CD45-positive cells (**Figure 1b**). To characterise these progenitors, we compared their gene expression profiles to blood-derived macrophages, macrophages and microglial-like cells derived from iPSC (**Figure 1c**). Expression of different lineage specific markers previously described (Abud et al., 2017; Butovsky et al., 2012) were analysed by RNA-sequencing. Results show that progenitors expressed high level of the CD34 hematopoietic stem cell marker and were RUNX1-high and MYB-low, a profile previously described as specific of yolk-sac progenitors (Ginhoux et al., 2010; Gomez Perdiguero et al., 2015; Kierdorf et al., 2013).

For macrophage generation, floating myeloid-like progenitors were plated onto coverslips in the presence of M-CSF. Within 7 days, cells acquired the characteristic elongated morphology of macrophages (and expressed CD45 and CD11b) (**Figure 1d**). Same morphologies were observed when these macrophages were cultured 7 more days on MNs (**Figure 1d**). To induce progenitor differentiation into microglial-like cells, progenitors were plated directly onto MNs in a medium containing interleukin-34 (IL-34), transforming growth factor 1 beta (TGF- β) and M-CSF (Easley-Neal, Foreman, Sharma, Zarrin, & Weimer, 2019; Ginhoux et al., 2010; Kierdorf & Prinz, 2019; Zoller et al., 2018). **Figure 1e** shows microglial-like cells expressing CD45 and CD11b, and illustrates morphological differences between progenitors matured alone or on MNs for 14 days. While the first ones displayed rather round shaped morphologies, progenitors matured onto MNs harboured ramified morphologies reminiscent of in vivo microglial cells interacting in their environment with MNs (Leyh et al., 2021).

Molecular characterization of iPSC-derived macrophages and microglia-like cells

Next, we performed a RNA-sequencing analysis on iPSC-derived macrophages cultured alone or onto MNs, iPSC-derived microglia-like cells cultured alone or onto MNs, as well as blood monocytes-derived macrophages (**Figure 2**). The three-dimensional principal component analysis (PCA) using all expressed genes showed efficient segregations of cells according to their status (**Figures 2a**). Interestingly, the first dimension allowed segregations of MP and blood monocytes-derived macrophages further away from microglia cultured alone and microglia and macrophages cultured over MNs. While the second and third dimensions segregated apart microglia cultured over MNs from macrophages cultured over MNs. Moreover, iPSC-derived macrophages cultured alone clustered close to blood monocytes-derived macrophages and segregated efficiently from

other cultures. These results are in agreement with recent reports that shed light on the importance of the neural environment for proper microglial maturation and differentiation (Abud et al., 2017; Haenseler et al., 2017). Although not reported in the literature, we observed that culture of macrophages over neurons also contribute to their maturation while maintaining a distinct transcriptomic profile from microglia cultured over neurons. Next, we performed a hierarchical clustering on microglia and macrophage-associated genes (Bennett et al., 2016; Butovsky et al., 2012; Douvaras et al., 2017; Haenseler et al., 2017; Hickman et al., 2013)(**Figure 2b**). We observed that microglia and macrophages groups clustered separately, and were further clustered depending on their culture environment. The transcriptomic profile of blood monocytes-derived macrophages appear to highly cluster with iPSC-derived macrophages mono-cultured. The heatmap shows that genes known to be expressed specifically in microglial cells including *P2RY12*, *P2RY13*, *ABI3*, *APOE*, *ENTPD1*, *OLFML3* and *CX3CR1*, had increased expressions in microglial-like cells cultured on MNs compared to the other cells and especially compared to microglial cells cultured alone, supporting previous findings which suggest a major role of neuronal contacts for microglia maturation (Haenseler et al., 2017; Takata et al., 2017).

Functional validation of iPSC-derived macrophages and microglia-like cells

To characterise iPSC-derived myeloid cells and distinguish macrophages from microglia-like cells, flow cytometry was used to analyse CD45 expression levels on the different iPSC-derived cell types. Results show that the highest membrane expression of CD45 was obtained on iPSC-derived macrophages mono-cultured, like their counterpart blood monocytes-derived macrophages, compared to iPSC-derived microglia (**Figure 3a**) (Ford, Goodsall, Hickey, & Sedgwick, 1995; Sedgwick et al., 1991). Another feature that allow the distinction between both cell types is the membrane expression of P2RY12. This receptor is a specific microglial marker (Bennett et al., 2016; Butovsky et al., 2012; Hickman et al., 2013) sensitive to ADP and necessary for microglial surveillance and sensing of the micro-environment (Gomez Morillas, Besson, & Lerouet, 2021). Quantitative RT-PCR confirmed the higher expression of mRNA encoding P2RY12 onto microglia matured on MNs compared to macrophages (**Figure 3b**). Then, their responses to ATP and ADP via the P2RY12 receptor were assessed by calcium imaging (**Figure 3c**). While both macrophages and microglial-like cells responded to ATP (not shown), only the microglial-like cells were able to respond to ADP. In addition, we also have characterised their phagocytic capacities, cytokine

secretion and migration. **Figures 3d and 3e** show that iPSC-derived cells were able to phagocytose either ovalbumin or human myelin. **Figure 3f** shows concentrations of cytokines in supernatants of macrophages or microglia-like cells cultured onto MNs. Finally, myeloid-like progenitors were injected into minibrains where they matured into Iba1 expressing macrophage/microglial cells and acquired characteristic morphologies (**Figure 3g**).

ALS macrophages and ALS microglia-like cells display trophic supports towards ALS MNs.

ALS and control iPSC were differentiated within 17 days into MNs as described previously (Guo et al., 2017; Maury et al., 2015). Seven days later, the MN medium was switched to specific media designed for co-cultures of MNs with macrophages or microglia-like cells. Cultures were analysed 11 or 14 days later. **Figure 4 and Figure 5** show results obtained for MN cultures either in the macrophage medium or in the microglia medium, respectively. In these culture conditions without macrophages or microglial cells, MN cultures accumulated cellular debris and showed alterations of neurite networks, especially in mutant MNs (**Figure 4a, 5a**). Quantifications of β 3-tubulin positive neurite network surfaces showed a two-fold decrease of neurite networks in C9orf72^{(G4C2)ⁿ} and SOD1^{N139D} MN cultures compared to control and TARDBP^{G348C} cultures in macrophages media (**Figure 4c**), and a two-fold decrease of neurite networks in TARDBP^{G348C} MNs compared to controls MNs cultured in microglia media (**Figure 5c**), showing that ALS MNs degenerated more rapidly than control MNs in the two types of media.

Next, to mimic the in vivo situation, ALS or control matched cultures were done between MNs and macrophages (**Figure 4b, Supplementary Figure 1a**) and between MNs and microglia-like cells (**Figure 5b, Supplementary Figure 2a**). Strikingly, we observed that cultures were healthier with no cellular debris and less bead-like structures on neurites, showing a trophic support of macrophages and microglia-like cells towards MNs (**Figure 4b, 5b**). Quantifications of neurite network surfaces in control, C9orf72^{(G4C2)ⁿ} and SOD1^{N139D} matched co-cultures were higher compared to control or mutant MNs cultures alone (**Figure 4c, Figure 5c**). When comparing the neurite network surface in matched ALS co-cultures, we observed a significant decrease in one of the four C9orf72^{(G4C2)ⁿ} patient patient - although networks were also affected for the other C9orf72 mutated patients -, SOD1^{N139D} and TARDBP^{G348C} macrophages cultures compared to controls (**Figure 4d**). While in microglia co-cultures, we witnessed only a significant decrease for one of

the 4 C9orf72^{(G4C2)ⁿ} patient - although networks were also affected for the other C9orf72 mutated patients - and in TARDBP^{G348C} cultures compared to controls (**Figure 5d**).

Finally, mixed cultures were established to assess the impact of ALS macrophages or ALS microglia-like cells towards control MNs (**Supplementary Figure 1b, Supplementary Figure 2b**). Clearly ALS macrophages and ALS microglia-like cells brought trophic supports to control MNs. Nevertheless, bead-like structures were still observed onto MN neurites when cultured with ALS myeloid cells, especially microglial-like cells, suggesting a dual effect from these mutant cells (intrinsic toxicity of these cells).

Taken together, these results show that ALS macrophages and ALS microglia-like cells were able to clear cellular debris in cultures of ALS MNs, to slow down neurite degeneration in ALS MN cultures, and demonstrated intrinsic toxicity towards control and ALS MNs.

ALS macrophages and ALS microglia-like cells in contact to ALS MNs have distinct alterations of the endolysosomal pathway.

To ask whether the endolysosomal degradation pathway was altered in ALS macrophages, we incubated mixed and matched co-cultures between control (Ctrl) or ALS (C9orf72^{(G4C2)ⁿ}, SOD1^{N139D}, TARDBP^{G348C}) MNs and macrophages with lysotracker, a marker of acidic compartments which label lysosomes. **Supplementary video 1** shows time-lapse images of lysosome dynamics in control and ALS matched co-cultures. Whereas in control macrophages the lysotracker labelled small individual organelles that moved into the cytoplasm, in ALS macrophages the staining showed enlarged organelles with decreased intracellular dynamics. **Figure 6a and Supplementary Figure 3** show that ALS macrophages had bigger organelles labelled with the lysotracker compared to control macrophages, both on matched cultures but also when ALS macrophages were co-cultured with control MNs, showing that defects in ALS macrophages were intrinsic. We then focused our analysis on two compartments of the endolysosomal pathway: LAMP1-positive lysosomes and EEA1-positive endosomes and we assessed whether their numbers and volumes were altered. **Figure 6b** shows examples of 3D confocal images from matched co-cultures between MNs and macrophages that were quantified according to EEA1 and LAMP1 immunostaining.

Numbers of LAMP1- and EEA1-positive vesicles per cell were quantified and results revealed higher number of Lamp1-positive vesicles in TARDBP^{G348C} macrophages compared to control.

While C9orf72^{(G4C2)ⁿ} macrophages demonstrated increased number of EEA1-positive vesicles compared to controls.(**Figure 7a, Figure 7b**)

Mean volumes of LAMP1-positive vesicles per cell were significantly increased in C9orf72^{(G4C2)ⁿ} macrophages and significantly reduced in SOD1^{N139D} macrophages, compared to control and TARDBP^{G348C} macrophages (**Figure 7c**). When single vesicle volume off all vesicles were analysed, quantification shows that the volume was significantly increased in C9orf72^{(G4C2)ⁿ} macrophages, while significantly decreased for SOD1^{N139D} and TARDBP^{G348C} macrophages compared to controls (**Figure 7e**).

Quantifications of mean volumes of EEA1-positive vesicles per cell shows significant increases in C9orf72^{(G4C2)ⁿ}, SOD1^{N139D} and TARDBP^{G348C} macrophages compared to control macrophages (**Figure 7d**). However, single vesicle volume of all vesicles showed decreased volume in TARDBP^{G348C} macrophages compared to controls (**Figure 7f**).

Taken together, these results showed that the endo-lysosomal pathway was perturbed in all ALS macrophages with a common mean increased of early endosomes volume per cell compared to controls.

Same experiments were conducted on co-cultures of MNs with microglia-like cells (**Figure 8**). In contrast to macrophages, control microglia-like cells were more intensely labelled by the lysotracker (**Figure 8a compared to Figure 6a**), revealing increased endo-lysosomal trafficking in microglia compared to macrophages. **Figure 8b** shows 3D confocal images from matched co-cultures between MNs and microglia-like cells that were quantified according to EEA1 and LAMP1 immunostaining. Quantifications showed that numbers of LAMP-1 vesicles per cell were decreased in ALS microglial-like cells compared to controls (**Figure 9a**), while numbers of EEA-1 vesicles were similar between control and ALS cells (**Figure 9b**). Moreover, LAMP1 vesicle volumes per cell (**Figure 9c, Figure 9e**) were reduced in ALS microglia-like cells compared to controls. In line with the increased lysotracker staining observed in microglia compared to macrophages, we noticed also that volumes of LAMP1 vesicles in control microglia-like cells were two-times bigger than the same vesicles in control macrophages (**Figure 7c**). Regarding EEA1, mean volumes per cell of EEA1 vesicles were identical between the different conditions. However, for individual EEA1 vesicles volumes we observed significant decreased volumes for all ALS mutants in comparison to control co-cultures, suggesting that the early endosomal pathway in ALS microglia may be perturbed (**Figure 9d, Figure 9f**).

In conclusion, our results show that the endolysosomal pathway is disturbed in ALS macrophages and microglia-like cells, with different alterations in numbers and volumes of early EEA-1 endosomes and LAMP-1 lysosomes, depending on the ALS mutation, suggesting a common altered mechanism with associated phenotypes that are mutation-dependent.

The different inflammatory secretion profiles of ALS macrophages and microglia-like cells upon contact with ALS or control MNs

To analyse whether the inflammatory secretomes of ALS and control macrophages or microglia-like cells were modified when they were in contact with mutant or control MNs, we collected supernatants of mixed and matched co-cultures. We measured 26 inflammatory markers at two time-points: after 3 days and after 11 days of co-cultures, a period corresponding to 8 days of aging of MNs in culture. In MNs cultured alone in macrophage or microglia media, at both time-points, each factor concentration was either below the detection level or detected at very low concentrations compared to those measured in co-cultures (**Supplementary Figure 4 and Supplementary Figure 5**).

In matched co-cultures between MNs and macrophages, some molecules secreted at detectable levels were unchanged in ALS and control co-cultures. They included interleukin-6 (IL-6), Monocyte Chemoattractant Protein 1 (MCP-1), soluble sCD40L and sCD130 (**Supplementary Figure 6a**). MCP-1 is a pro-inflammatory chemokine with a chemotactic activity for monocytes and basophils (Martinez et al., 2020). IL-6 response can be mitigated: IL-6 anti-inflammatory signalling is mostly mediated via the membrane bound IL-6 receptor (IL-6R), while the pro-inflammatory signalling is mostly dependent on the soluble IL-6R (sIL-6R)(Scheller, Chalaris, Schmidt-Arras, & Rose-John, 2011). sCD40L and sCD130 are both soluble receptors. sCD40L is a member of the TNF family with pro-inflammatory effects (Takada, Yu, Shimoda, & Takada, 2019), while sCD130 is a master regulator of cytokine-mediated inflammatory responses and has a main anti-inflammatory role by acting as an inhibitor of sIL-6R (Jostock et al., 2001). Interestingly, we observed that the initial macrophages response was mediated by chemotaxis signalling as well as IL-6, which could both have anti- and pro-inflammatory functions. With MNs aging, we observed increased sCD130 release, possibly to counteract the increased level of IL-6 secretion and prevent pro-inflammatory signalling. Besides this, the concentrations sCD40L remained mostly stable between day 3 and day 11. Taken together, these results demonstrate a

temporality in secretion of the different factors, suggesting a common secretory signature between ALS and control macrophages upon contact with ALS or control MNs. When compared with co-cultures between MNs and microglia-like cells, only two molecules were secreted as soon as day 3 and had stable secretions until day 11 (**Supplementary Figure 7a**). Similarly to macrophages, MCP-1 was secreted early on and prolonged over time, suggesting that microglia-like cells sustained over time the chemotactic signal to attract monocytes and basophils. The other molecule commonly secreted was PTX3 (see below). Secretion concentrations of both MCP-1 and PTX3 were comparable between the different ALS forms, suggesting that these two factors represent a generic response of microglia to MNs aging in cultures.

Regarding the factors, which were differentially deregulated between control and ALS mutants macrophages, seven factors were identified: two interleukins (IL-10 and IL-8), PAI-1 also named Serpin Family E member 1 (SERPINE1), Pentraxin-3 (PTX3), the soluble Tumor Necrosis Factor Receptors I and II (sTNFR-I and sTNFR-II) and sCD25 (**Figure 10 and Supplementary Figure 6b**). At day 3 only the two interleukins had significantly altered secretion profiles: IL-10, that increased in C9orf72^{(G4C2)ⁿ} and SOD1^{H44R} co-cultures, and IL-8 that was differentially deregulated with an increased concentration in C9orf72^{(G4C2)ⁿ} and a decreased one in TARDBP^{G348C} matched cultures. These results show that ALS macrophages were rapidly activated upon contact with ALS MNs. IL-10, as an anti-inflammatory cytokine with pleiotropic effects in immune-regulation, could be secreted to dampen an inflammatory response, while IL-8, as a major chemotactic factor that attracts T-cells, neutrophils and basophils, could be secreted to attract these cells to help resolving inflammation. At day 11 whereas IL-10 and IL-8 secretion levels were reduced, five factors showed either increase or decrease secretion patterns. Among them, PAI-1 or Serpin Family E member 1 (SERPINE1) and Pentraxin 3 (PTX3) secretions were significantly increased in all ALS forms (C9orf72^{(G4C2)ⁿ}, SOD1^{H44R} and TARDBP^{G348C}). PAI-1 secretion was already increased at day 3 in TARDBP^{G348C}, albeit at a lower level. PAI-1 is the main physiological inhibitor of plasminogen activators and belongs to the family of serine protease inhibitors (Sillen & Declerck, 2021). Interestingly, PAI-1 mRNAs and its receptor LRP1 were detected by RNA sequencing in iPSC-derived MNs (Lefebvre-Omar et al.*). As PAI-1 secretion was barely detected in cultures of MNs alone, our results suggest that either ALS MNs or ALS macrophages or both, increased their secretion of PAI-1 when they are in contact.

The profile of secretion in matched co-cultures of MNs and microglia-like cells was rather different than the one of macrophages. When comparing ALS to control matched co-cultures of MNs with microglia-like cells (**Figure 11**), the IL-10, IL-8 and IL-6 secretion profiles were reminiscent of the ones in MNs and macrophages co-cultures, suggesting a similar early response to an inflammatory environment from the mutant microglia-like cells. However, PAI-1 was not increased anymore in C9orf72^{(G4C2)ⁿ} co-cultures, yet was increased in SOD1^{H44R} and TARDBP^{G348C} co-cultures. Moreover, the multifunctional cytokine transforming Growth Factor beta 1 (TGFβ1) already known to be deregulated in ALS (Galbiati et al., 2020) was found secreted at day 3 only in SOD1^{N139D} co-cultures and in both SOD1^{N139D} and TARDBP^{G348C} co-cultures at day 11. This cytokine was not detected in macrophage co-cultures, suggesting a specificity of microglial cells.

Finally, at day 11, secretions of different soluble receptors including the two receptors of the pro-inflammatory TNF family, sTNFR-I and sTNFR-II, the IL-2 receptor subunit alpha, sCD25, and sCD130, a soluble IL-6 receptor, were variable from one ALS form to the other (**Supplementary Figure 6b and Supplementary Figure 7b**). As soluble receptors could have agonist or antagonist functions, it remains difficult to conclude on their exact contributions to MN degeneration in co-cultures.

To assess how MNs influenced secretomes of mutant macrophages and mutant microglial-like cells, these latter were cultured onto control MNs (**Figure 10, Figure 11**). For some factors, the cultures with control MNs did not change secretion concentrations, suggesting that the myeloid cells were intrinsically primed. For some others, significant differences were observed between matched and mixed cultures, suggesting that mutant MNs could influence myeloid cell behaviours. Interestingly, we found that PTX-3 concentrations in C9orf72^{(G4C2)ⁿ}, SOD1^{H44R} and TARDBP^{G348C} macrophages cultured onto control MNs were significantly lower than concentrations in ALS matched cultures and closer to concentrations in control matched cultures. In some other situations, concentrations in mixed cultures of mutant myeloid cells with control MNs were equivalent to those in ALS mixed cultures, suggesting that ALS MNs and both types of myeloid cells influenced the secretomes.

Taken together, these results show that secretion profiles of macrophages and microglia-like cells shared similar inflammatory responses involving IL-10, IL-6 and IL-8 cytokines and several soluble receptors that could have agonist or antagonist effects. The main differences between ALS

macrophages and ALS microglia-like cells were their specific PTX3 and TGF- β 1 secretions in responses to MN degeneration, respectively. Finally, PAI-1 secretion was deregulated in both myeloid cell types and thus appeared as a possible marker of the inflammatory responses in all ALS forms.

Transcriptomic analysis reveal distinct profiles between ALS macrophages and microglia-like cells.

Transcriptomic analyses were conducted on macrophages and microglia-like cells isolated at d11 from (controls and ALS) matched co-cultures. Cells were negatively sorted from the co-cultures to avoid any activation by beads and we checked by quantitative PCR that isolated cells were myeloid cells. We observed a total of 493 and 1654 dysregulated genes in C9orf72^{(G4C2)ⁿ} macrophages and microglia respectively compared to controls; 1827 and 15 genes dysregulated in SOD1^{N139D} macrophages and microglia respectively compared to controls; and 3913 and 586 genes dysregulated in TARDBP^{G348C} macrophages and microglia respectively compared to controls (**Figure 12a**). Gene Set Enrichment Analysis (GSEA) using several human databases revealed common deregulated pathways. We observed different main cellular functions that were deregulated in ALS microglia-like cells compared to ALS macrophages. Very interestingly, **Figure 12b** shows cytokine signalling pathways and **Figure 12c** shows proteostasis pathways including endosomal, lysosomal, proteasomal and autophagy pathways, highly dysregulated in all ALS mutant microglia-like cells compared to controls, and also compared to ALS macrophages. These results confirm that ALS microglial-like cells and ALS macrophages reacted very differently when in contact to degenerating MNs and have thus to be considered as two different cell types. Analysis of ALS macrophages show that dysregulations existed in the same pathways (**Figure 12a and Figure 12b**) to lower levels than in ALS microglia-like cells. In contrast to ALS microglial-like cells, we observed differences between ALS forms with only one common dysregulated pathway, the late endosomal microautophagy. The autophagy pathway was only altered in C9orf72^{(G4C2)ⁿ} macrophages compared to controls, while SOD1^{N139D} and TARDBP^{G348C} macrophages demonstrated mostly dysregulations in pathways linked to endosomes and lysosomes compartments (**Figure 12c**). Finally, we observed common altered pathways including inflammasome and complement pathways, as well as production of ROS and RNS (**Figure 12d**), suggesting an underlying inflammatory phenotype in both myeloid cell types upon culture over

mutant MNs. Interestingly, the “diseases of the immune system” pathway was shown also to be commonly affected in ALS macrophages and microglia. Finally, the neuronal pathway was highly deregulated specifically in ALS microglia-like cells, suggesting that the latter might bear specific neuronal functions, which could be recapitulated in our co-cultures conditions.

Discussion

With the present study, our aim was to better decipher the roles played by ALS macrophages and microglial cells in the pathology and in particular their trophic or toxic supports towards ALS MNs during degeneration. There is evidence that both types of myeloid cells are activated in ALS patients, but their respective contributions remain unknown. Pioneered studies showed the toxic role of macrophages and/or microglia in SOD1 mouse models (Beers et al., 2006; Boillee et al., 2006) and recently Chiot et al. demonstrated in the same model that both macrophages and microglia reacted differently to MN degeneration (Chiot et al., 2020). In a therapeutic perspective to slow down disease progression in all ALS forms, being able to target one cell type rather than the other is a crucial point to consider. Macrophages being located at the periphery, they appear easier targets than microglial cells in the CNS. This being, more investigations need to be conducted to understand the roles of these inflammatory cells in different ALS forms and to define possible targets. In this context, the iPSC technology offers a unique opportunity to model interactions between MNs and inflammatory cells. With this objective in hand, we compared in a similar experimental context iPSC derived macrophages or microglial from patients with different ALS forms. Firstly, we developed a differentiation protocol allowing the generation of iPSC derived macrophages or microglial cells with their own specific characteristics, and resulting from the same pool of progenitors. Secondly, when co-cultured with ALS MNs, although we could observed trophic support mediated by the clearance of cellular debris, yet we noticed alterations in their endolysosomal pathway. Thirdly, we identified distinct secretory profiles of ALS macrophages and ALS microglia when co-cultured with MNs and interestingly that these profiles evolved differentially with time in culture. Finally, our transcriptional analysis strengthened our previous observations, revealing similarities but also differences in dysregulated pathways between ALS macrophages and ALS microglial-like cells (both cultured onto ALS MNs and compared to cells isolated from matched control co-cultures), linking inflammatory responses to defective degradation pathways in these innate immune cells during ALS pathology.

During the inflammatory response, first line effectors are innate immune cells. In ALS patients both macrophages at the periphery along axonal nerves and microglia close to MN soma were shown to be activated. The two major responses to MN suffering are the secretion of inflammatory mediators and clearance of cellular debris mediated by phagocytosis to resolve inflammation. In our study, we witnessed deregulation in both responses in co-cultures of ALS macrophages or ALS microglia onto ALS MNs, compared to match control cultures.

First obvious consequences of co-culturing ALS macrophages or microglial-like cells onto ALS MNs were the trophic supports brought by the myeloid cells to MNs and the clearance of debris. Phagocytes from the innate immune system are characterised by their clearance functions, which contribute to the maintenance of cell homeostasis. Upon phagocytosis and formation of early endosomes, the cargo can be recycled to the plasma membrane, or transit through the ubiquitin proteasome or the autophagic pathways for degradation. The autophagy-lysosomal system includes three different type of autophagic degradation. Each one acts through a distinct molecular mechanism, but all of them ultimately depend on functional lysosomes. The major involvement of lysosomes in multiple cellular functions (signaling, immunity, adhesion, migration) has been put to light in the past years and demonstrate the importance of their well-functioning for maintaining cellular homeostasis (Ballabio & Bonifacino, 2020). Growing evidence indicates that not only lysosomal storage diseases but also neurodegenerative diseases may involve lysosomal dysfunctions (Ballabio & Bonifacino, 2020). To analyse if degradative pathways could be altered in ALS innate immune cells, we stained the latter with antibodies directed against LAMP1 and the early endosome EEA1 marker. In ALS, evidences of altered proteostasis in innate immune cells came from studies of mutant C9orf72 models. In C9orf72 KO mice, bone marrow derived macrophages and microglia were shown to have increased lysotracker and LAMP1 staining (O'Rourke et al., 2016). These characteristics were also observed in C9orf72^{(G4C2)ⁿ} patients, LAMP1 accumulations were observed in post-mortem tissues however were absent in sporadic cases (O'Rourke et al., 2016). Yet Zondler et al. showed that monocytes from sporadic ALS patients exhibited decelerated phagocytosis and impaired endosome-to-lysosome vesicle trafficking (Zondler et al., 2016). In our experiments, we observed decreased number of LAMP1-positive vesicles and decreased mean volumes of LAMP1-positive vesicles per cell in C9orf72^{(G4C2)ⁿ}, SOD1^{N139D} and TARDB^{G348C} microglial-like cells in matched cultures. In parallel, we also

observed decreased individual EEA1-positive vesicle volumes. Taken together, these results suggest that the endolysosomal pathway is affected in ALS microglial-like cells possibly affecting their phagocytic capacities. In ALS macrophages, the picture is opposite. While numbers of LAMP1 vesicles increased in TARDBP^{G348C} macrophages, their numbers were not modified in C9orf72^{(G4C2)ⁿ} and SOD1^{N139D} cells, and regarding mean volumes of LAMP1-positive vesicles per cell, whereas they increased in C9orf72^{(G4C2)ⁿ}, they decreased in SOD1^{N139D} and remained constant in TARDBP^{G348C} macrophages. For EEA1-positive vesicles, only C9orf72^{(G4C2)ⁿ} numbers increased, while mean volumes of EEA1-positive vesicles per cell increased in C9orf72^{(G4C2)ⁿ}, SOD1^{N139D} and TARDBP^{G348C} macrophages in matched cultures. Overall, these results support an overloading of ALS macrophages compared to controls. Taken as a whole, although macrophages and microglia are able to clear cellular debris and bring trophic support to MNs cultures, we witness defects in endolysosomal pathways in both cell types suggesting that compensatory mechanisms may operate in order to respond to specific defects in phagocytic capacities.

To analyse trophic or toxic supports of ALS innate immune cells in our cultures, we quantified secretions of 26 inflammatory molecules. Among them, only 11 were significantly expressed in one and/or the other culture conditions. Some factors were expressed without differences between ALS or control cultures (considered as canonical responses of myeloid cells), while some others significantly varied when ALS cultures were compared to control ones. At the early time point, when ALS MNs appeared healthier in contact to myeloid cells compared to culture of MNs alone, anti-inflammatory cytokine, IL-10, was highly secreted, as well as two main chemoattractant factors: IL-8 and MCP-1. The upregulation of IL-10 suggest an early specific anti-inflammatory response from innate immune cells upon contact with mutant MNs. Moreover, IL-10 could be responsible for the subsequent increased expression of sTNFR-I and sTNFR-II at later time point. In fact, these two soluble receptors able to sequester TNF- α and prevent its inflammatory effect on cells (Cereda et al., 2008; Hart, Hunt, Bonder, Watson, & Finlay-Jones, 1996). On the other hand, while IL-8 secretion appeared variable between the different ALS forms, MCP-1 was secreted by all control and ALS co-cultures containing microglial cells or macrophages. Our results are in agreement with meta-analysis of ALS patient's biofluids, in which MCP-1 was found to be most of the time increased in the CSF and detected at stable levels in the blood (Chen, Hu, Cao, Liu, & Cheng, 2018; Hu et al., 2017), suggesting a maintenance of the signal intended to attract leukocytes

and in particular macrophages to resolve the injury. Besides these factors, IL-6 was also found expressed early on in co-cultures. IL-6 is a pleiotropic cytokine with both anti- and pro-inflammatory functions (Hu et al., 2017; Tortelli et al., 2020). Its secretion levels was higher at the early time point and decreased with MN aging. In parallel we observed increased secretion of sCD130, a soluble form of the signal transducer gp130 interacting with IL-6 and its receptor (Jostock et al., 2001; Scheller et al., 2011) which could block the pro-inflammatory action of IL-6. Altogether, these results suggest that a “classical” inflammatory response is shared by mutant and control macrophages and microglia in reaction to aging MNs, which appeared to balance in favor of an anti-inflammatory reaction.

Besides these quite “classical” patterns of secretions of known inflammatory cytokines, two molecules that may have anti- or pro-inflammatory effects presented altered secretory profiles in mutant ALS cells compared to controls. One secreted molecule detected only in ALS cells co-cultures, PAI-1, appeared particularly interesting. Whereas we were unable to detect its secretion in control co-cultures, its level increased in C9orf72^{(G4C2)ⁿ}, SOD1^{H44R}, TARDBPG^{348C} matched macrophages and MNs co-cultures, and in SOD1^{N139D}, TARDBP^{G348C} matched microglial-like cells and MNs co-cultures, suggesting a role of PAI-1 secretion in the MN fate in ALS. Interestingly, we could prevent the secretion of PAI-1 when mutant cells were cultured onto control MNs, suggesting that the ALS MNs promote an environment which support PAI-1 secretion. PAI-1 is not only an inhibitor of plasminogen activators but also through its inhibition of plasmin activation, it is involved in various processes including tissue remodeling, cell migration and inflammation (Sillen & Declerck, 2021). In brain tissue, PAI-1 was shown to be induced by a wide range of pro-inflammatory mediators including IL-6 and was shown to protect neurons by preventing disintegration of neuronal networks and maintaining or promoting neuroprotective signaling through the MAPK/ERK pathway, independently from its function as a proteinase inhibitor (Soeda et al., 2008). Moreover, PAI-1 was shown to promote microglial cell migration and to modulate their phagocytic activity (Jeon et al., 2012). Whether PAI-1 has the same actions on macrophages remains to be determined. Taken together, this suggests that PAI-1 could be secreted in our ALS co-cultures in response to MN suffering and that it could help slowing down MN degeneration. However, PAI-1 was also shown to have negative contributions in several diseases including multiple sclerosis (MS), Alzheimer (AD) and Parkinson (PD) diseases. In MS

and in AD, elevated PAI-1 levels impaired clearance of fibrin deposits, therefore contributing to axonal damages, and degradation of β -amyloid, contributing to deposition of $A\beta$, respectively (Gveric, Herrera, Petzold, Lawrence, & Cuzner, 2003; Jacobsen et al., 2008; Oh, Lee, Song, Park, & Kim, 2014; Rodriguez-Lorenzo et al., 2020). In PD, an inflammatory loop was suggested to operate between increased α -synuclein aggregation, the inflammatory microglial response and increased PAI-1 levels (Pan et al., 2018; Reuland & Church, 2020).

In ALS, there is no report to our knowledge about the role of PAI-1 in protein inclusion clearance or inflammation. Our study is the first one suggesting a link between increased PAI-1 levels, activation of macrophages and microglial-like cells and MN degeneration as a common mechanism between several ALS forms. A scenario could be that PAI-1, induced by IL-6, promoted at first MN survival, but with time its accumulation led to alterations of phagocytic activities of myeloid cells, accumulation of cellular debris, within a non-resolving loop, as described in PD. In ALS, a previous study showed an increase in PAI-1 concentrations in the blood of patients (Ngo et al., 2015) with no correlation with disease duration or the ALSFRS-R score. However, like in the majority of studies of ALS patient's biofluids, the population was highly heterogeneous that any conclusion has to be taken with a grain of salt. Because of its contribution to a wide variety of pathological processes, diverse approaches already exist to inhibit PAI-1 and this will be interesting in future experiments to target PAI-1 in our experimental model to see whether we can observe significant beneficial effects (Sillen & Declerck, 2021).

Another secreted molecule of interest is PTX3, as it was increased in C9orf72^{(G4C2)ⁿ}, SOD1^{H44R}, TARDBPG^{348C} matched macrophages and MNs co-cultures, while being secreted at stable levels in ALS matched microglial-like cells and MNs co-cultures. PTX3 has complex immunological functions (Wu et al., 2020). As its production by macrophages is induced by pro-inflammatory cytokines, PTX3 is known as an acute phase protein produced rapidly at local sites of inflammation in order to resolve inflammation (Shiraki et al., 2016). PTX3 was also shown to contribute to neurogenesis after brain injury in mice (Zhou et al., 2020). However, like PAI-1, besides its protective role, evidences suggest that PTX3 could also have detrimental effects (Magrini, Mantovani, & Garlanda, 2016). While in some inflammatory processes PTX3 was shown to play protective roles, its induction was also associated to tissue damage in pathological contexts. These

differences were attributed to the tissue context, cellular source and levels of secretion of PTX3 (Magrini et al., 2016). Nevertheless, PTX3 is seen today as a potential diagnostic and prognostic marker of inflammation (Magrini et al., 2016). Overall, it remains difficult to conclude about the role of PTX3 in ALS. A recent study analyzing the transcription profile of monocytes from ALS patients identified PTX3 among the deregulated genes and solely in monocytes from rapidly progressing patients (Zhao et al., 2017). Their result is in agreement with our results as in SOD1^{N139D} patient's cells the level of PTX3 was null in macrophages and MN co-cultures, while the level of PTX3 secretion increased in the patient mutated in SOD1^{H44R}, a patient who has a rapidly progressing form of ALS compared to the other patient (Supplementary Table 1).

Although commonalities of secretory profiles were observed between the two innate immune cell types, some responses were specific to each cell type. For example, TGF- β 1 was only detected in microglial-like cell cultures and was induced in SOD1^{N139D}, in SOD1^{H44R} and TARDBP^{G348C} mutant co-cultures. TGF- β 1 is a pleiotropic cytokine known to be dysregulated in ALS with different roles in early and late disease stages which are concentration dependent (Galbiati et al., 2020). On the other side, sCD40L was only detected in macrophage cell cultures, without any variation. sCD40L is a member of the TNF family with pro-inflammatory effects (Takada et al., 2019). This soluble ligand was also detected among genes deregulated in monocytes of patients with rapidly progressing forms (Zhao et al., 2017). In agreement with our results, sCD40L was secreted at a higher level in co-cultures of SOD1^{H44R} macrophages and MNs, compared to SOD1^{N139D} co-cultures, suggesting that in vitro model can recapitulate some features of in vivo patient's cells.

Besides these specific responses of myeloid cells in co-cultures with MNs, we noticed that many inflammatory factors were not detected in our analysis either because they were not secreted in our culture conditions or because the detection levels of the assay were too low. They included IL- β , IL-18, IL33, IL-12p70, IL-17A, IL-23, IFN γ , IFN α 2, TNF α , sTREM1, CXCL12, sST2, sRAGE and CXCL1. This advised us that either the environment did not stimulate their secretion by macrophages, microglial-like cells nor MNs or possibly that in ALS patients the reported presence of these factors originate from other secretory immune cells than macrophages or microglia (Chen et al., 2018; Hu et al., 2017).

As a whole, our transcriptomic analysis supported our observed cellular and molecular alterations in ALS mutants compared to controls. Indeed, GSEA analysis revealed that both ALS macrophages and microglia shared same altered pathways especially those involved in cytokine signaling and degradation pathways. Interestingly, endo-lysosomal pathways were less deregulated in ALS macrophages compared to ALS microglial cells, suggesting that these defects may lead to compensatory mechanisms in microglia-like cells in order to support neuronal survival until a point where the response shifts to a toxic profile.

Taken together, our results show that ALS macrophages and ALS microglial-like cells adopted a trophic response towards suffering MNs. However cytokine profiles and alterations of the endo-lysosomal pathways suggest that the trophic response could rapidly change to a deleterious environment with the overload of the degradative pathways and the secretion of molecules like PAI-1 and PTX3, which are known to be beneficial and detrimental in a context-dependent manner, could take the advantage leading to MN death. As inflammation and proteostasis regulate each other, when both sustained inflammation and chronic proteostasis alteration met, each one could potentiate the other, increasing cell vulnerability.

In conclusion, we have set up co-culture models of human MNs and innate immune cells and we have shown that these models are relevant to study interactions between MNs and macrophages and microglia. In ALS, other immune cell types are involved and protocols now exist to generate those cells from iPSC (Liu et al.*). Studying interactions of innate immune cells with lymphocytes, dendritic cells and mast cells, for examples, could bring new insights into the chronology of inflammatory events in ALS.

Methods

Human cells. A fibroblast collection from ALS patients was established to generate iPSC. Skin biopsies were proposed to french ALS patients, in collaboration with the French National Referral Center for ALS at the Pitié-Salpêtrière hospital (Paris, France) and the Institut Pasteur (project promoter) (Paris, France). The protocol was validated by the Agence Française de Sécurité Sanitaire des Produits de Santé (AFSSAPS) and a Comité de Protection des Personnes (CPP) (CPP

Ile de France VI; protocol 2010-33-ALSCELL). Fibroblasts from the TARDBP gene mutated patient were obtained from the Erasmus Hospital (Brussels, Belgium) after informed consent of the patient and approval of an ethical committee (CPP Ile de France II, DC 2011-534). Patients signed explicit consents. For control subjects, skin fibroblast cultures were obtained from the Centre de Ressources Biologiques (CRB) of Lyon (France). A statement of all biological samples was made according to french laws formulated by the Research Ministry. The list of iPSC clones is in Supplementary Table 1. Fibroblasts from patients mutated in SOD1^{N139D}, ¹C9ORF72^{(G4C2)ⁿ}, ²C9ORF72^{(G4C2)ⁿ} and from the control 69 (69 years old) were reprogrammed with the integration-free CytoTune-iPS 2.0 Sendai Reprogramming Kit (ThermoFisher Scientific), as described previously (Genin et al., 2019). For the patient mutated in TARDBP^{G348C}, reprogramming of fibroblasts was done with integrative retroviral vectors as previously described (Lemonnier et al., 2011), except that fibroblasts were reprogrammed with 5 vectors encoding OCT4, SOX2, KLF4, C-MYC and NANOG. Other clones were bought either from the European Collection of Authenticated Cell Cultures (ECCAC) or the Catalog of the NINDS Human Cell and Data Repository (NINDS). Isogenic controls were either bought from same collections or generated by the ICM CELIS internal platform. iPSC were cultured on Geltrex (ThermoFisher Scientific), passaged with 0.5mM UltraPure™ EDTA pH 8.0 (ThermoFisher Scientific), and maintained in Essential 8-Flex medium (ThermoFisher Scientific).

Differentiation of iPSC into MNs. MNs differentiation was performed as described in Lefebvre-Omar et al.*. Briefly, iPSC clones were dissociated within StemPro™ Accutase™ (ThermoFisher Scientific). $2 \cdot 10^5$ cells/ml were suspended into a Neuronal Basic Medium (NBM) composed of 1:1 DMEM/F-12/Neurobasal™ (ThermoFisher Scientific), N2 (ThermoFisher Scientific), B27™ supplement minus vitamin A (ThermoFisher Scientific), L-Ascorbic acid (0.01 μM, Sigma), and antibiotics, supplemented with SB431542 (20μM, Tocris Bioscience, Bristol, UK), LDN-193189 (0.2μM, Stemgent) and CHIR99021 (3μM, Stemgent). Embryoid Bodies (EBs) were formed within one day, and 2 days later the NBM was supplemented with retinoic acid (0.1μM, Sigma) and SAG smoothed ligand (0.5μM, Enzo Life Sciences, NY, USA) until day 14. CHIR99021 was removed at day 2, while SB431542 and LDN-193189 were removed at day 4. BDNF, GDNF (10ng/mL, Miltenyi Biotec) were added at day 7. At day 9, DAPT (10μM, Sigma) was added and EBs were dissociated the next day with Trypsin-EDTA (ThermoFisher Scientific). Dissociated

single cells were seeded onto coverslips coated either with Polyethyleneimine (PEI) solution (2mg/ml, Sigma) or Poly-ornithine (100µg/ml, Sigma), and laminin (20µg/ml, Sigma). Between day 14 and day 17, cells were incubated in NBM with DAPT, BDNF and GDNF. From day 18 and until the end of each experiment, DAPT was removed from NBM, while CNTF and IGF1 were added (10ng/mL, Miltenyi Biotec). Half of the medium was changed every other day. Cultures were maintained for 25 days after dissociation.

Differentiation of iPSC into macrophages and microglial-like cells. The protocol was adapted from two different reports (Douvaras et al., 2017; Haenseler et al., 2017; Pandya et al., 2017; Yanagimachi et al., 2013). iPSC were dissociated with StemPro™ Accutase™ and resuspended into the mTesR1 medium (Stem Cell Technologies), at a density of 40×10^5 cells per T25 flask. The medium was supplemented with hES Cell Cloning and Recovery Supplement (ReproCell Europe). The day after the medium was renewed and bone morphogenetic protein 4 (BMP4 at 80 ng/mL) was added. Four days later, the medium was switched to the StemPro™-34 SFM medium supplemented with vascular endothelial growth factor (VEGF at 80 ng/mL), stem cell factor (SCF at 100 ng/mL), and fibroblast growth factor 2 (FGF2 at 25 ng/mL). Two days later, the medium was supplemented with SCF (50 ng/mL), Fms-like tyrosine kinase 3 ligand (FL3t at 50 ng/mL), macrophage colony-stimulating factor (M-CSF at 50 ng/mL), Interleukin-3 (IL3 at 50 ng/mL) and thrombopoietin (TPO at 5 ng/mL). Medium was changed 5 days later. Four days later, the StemPro™-34 SFM medium was supplemented with FL3t (50 ng/mL), M-CSF (50 ng/mL) and granulocyte macrophage colony-stimulating factor (GM-CSF at 25 ng/mL). All cytokines were from Miltenyi Biotec. Three days later differentiated cells in suspension were harvested and we performed harvests every 3-4 days until harvest 4.

To induce macrophage maturation, harvested cells were plated at a density of 0.5×10^5 cells per cm² on coverslips coated with poly-D-lysine (10 µg/mL; Sigma) in RPMI1640 medium (ThermoFisher Scientific) supplemented with 10% fetal bovine serum (ThermoFisher Scientific), M-CSF (100 ng/ml), non-essential amino acid (ThermoFisher Scientific), and sodium pyruvate (ThermoFisher Scientific). Half of the medium was changed once every 3 days and cytokine concentrations were doubled to obtain similar final concentrations. Macrophages cultures were detached seven days later and plated onto MNs in the macrophage medium. To induce microglia differentiation, progenitors were directly plated onto MNs (21 days of differentiation from iPSC) at a density of

0.5x10⁵ cells per cm² in Advanced DMEM/F-12 medium (ThermoFisher Scientific) supplemented with N2, β-mercaptoethanol, interleukin-34 (IL-34 at 100 ng/ml), transforming growth factor beta1 (TGF-β1 at 50 ng/ml) and M-CSF (25 ng/ml).). Half of the medium was changed once every 3 days and cytokine concentrations were doubled to obtain similar final concentrations.

Macrophages and microglia isolation by negative magnetic cell sorting. Co-cultures with MNs were treated with StemPro™ Accutase™ for 5 minutes. Flushing of cells into dishes was sometimes required to ensure total cell detachment. Cells were then labelled with p75NTR microbeads and magnetically sorted according to the supplier (Miltenyi Biotech). This allowed the positive sorting of the fraction containing MNs expressing the p75 neurotrophic receptor (Toli et al., 2015) and the negative sorting of macrophages or microglia-like cells. Cells were then counted, pelleted and frozen.

Immunocytochemistry. Medium was not removed from wells and paraformaldehyde (PFA) (Euromedex) was gently added to have a 4% final concentration. Incubation lasted 10 minutes at room temperature (RT). Then cells were washed in phosphate buffered saline (PBS). Saturation was done with 5% normal goat serum (ThermoFisher Scientific) with or without Triton-X100 (0.1%) for 15 minutes at RT. Primary antibodies were added and cells were incubated overnight at 4°C. Secondary antibodies were added for 1 hour at RT. Nuclei were stained with Hoechst H33342 (ThermoFisher Scientific). Slides were mounted with Fluoromount G (Southern Biotech). Primary antibodies include mouse anti-β3-tubulin (1/500, Covance), anti-CD45 (IgG1, 1/200, Biolegend), anti-CD11b (Rat biotinylated, 1/500, BD pharma), anti-IBA1 (rabbit, 1/500, Wako), anti-LAMP1 (IgG1, 1/250, Santa Cruz biotechnology), anti-EEA1 (Rabbit, 1/500, Cell Signaling Technology). Secondary antibodies were goat anti-mouse IgG1 AlexaFluor® 488, AlexaFluor® 555, AlexaFluor® 647, goat anti-mouse IgG2a AlexaFluor® 555, AlexaFluor® 647, goat anti-rabbit AlexaFluor® 488, AlexaFluor® 555, AlexaFluor® 647 (ThermoFisher Scientific). The lysotraker was from ThermoFisher Scientific and used according to the supplier. For phagocytosis, cells were incubated with Ovalbumin-FITC (ThermoFisher Scientific) or tagged-FITC myelin (a gift from Dr Zujovic) for 30 minutes at 37°C or 4°C (as a control). Cells were then fixed with 4% PFA and immune-labelled with anti-β3-tubulin and CD45 as described above.

Cytokines secretion analysis. Supernatants were collected and centrifuged at 4°C, 300g, 7 minutes. Pellets of cell debris were discarded and supernatants were frozen at -80°C. Two bead-based multiplex assays were used to measure 26 inflammatory factors (LEGENDplex human inflammation panel 1 and panel 2)

Each experiment was done with replicates. Fluorescence emission was measured using Macsquant Analyzer 10 Flow Cytometer and data analysis were made with the Legendplex analyser software.

Flow cytometry. Floating progenitors were collected at each harvest. Cells were counted and resuspended into PBS with EDTA (2mM) and plated into 96 conic bottom plates. Viability staining was done without any serum. Cells were incubated with Viability Dye eFluor (1:200; ThermoFisher Scientific) at 4°C during 20 minutes. The plate was then centrifuged at 4°C, 300g, for 3 minutes. Cells were incubated with FCR blocker diluted in PBS with EDTA supplemented with 2,5% of FBS (ThermoFisher Scientific) in half of the final volume at 4°C for 30 minutes. Anti-CD45-PE-Cy7 and its isotype were added to each well (2µL/10⁶ cells) and cells were incubated at 4°C for another 30 minutes. Cells were washed 3 times with PBS EDTA supplemented with 2,5% of FBS. Analysis was realized on a MACSquant and data analysed using Flowjo software.

Calcium imaging. Cells were plated into 8 chambers glass bottom microslides, washed with HBSS+ (with Ca²⁺ and Mg²⁺) and incubated with Fura-2, AM (ThermoFisher Scientific) at a final concentration of 1µM diluted into HBSS+ for 40 minutes at room temperature. Cells were then washed once rapidly with HBSS+ and then let incubated 15 min with HBSS+. Prior to imaging, HBSS+ was removed and replaced by HBSS without Ca²⁺ Mg²⁺. Cells were imaged on Nikon TI Eclipse microscope at 20X S-Plan-Fluo 0.40 using Hamamatsu ORCA-D2 Dual CCD Camera. HC Image Software (Hamamatsu Photonics) was used for to manage time lapse imaging. Sequences were taken over 2 minutes with an image taken every second were acquired. Fluorescent intensities are measured at two wavelength 340nm 380nm. ATP (200 µM; Cayman) or ADP (100 µM; Cayman) is added after 20 images.

Background noise was measured and subtracted from sequence. Delta F/F were calculated for each sequences and time curve was generated with Excel software.

Migration assay. Cells were plated into culture-insert 2 well micro dish 35mm (iBidi). Few minutes before starting time-lapse recordings, the insert was removed using dissection pliers. Cells were imaged using Axio Observer 7 with 40X LD PlanNeofluar 0.75 NA, using Hamamatsu Orca Flash 4.0 camera. Image recordings were done using Zen Software, every 5 or 10 minutes over 6 to 24 hours. Images were subsequently analysed using image J.

Neuritic network quantification. Images were acquired using Axioscan Z1 at 20X magnification with a Hitachi 3CCD color camera. All microscope and camera settings were identical for the different acquired images. Images were further analysed with Fiji Software using the thresholding function and was applied similarly on all the different images. Images were converted to 8-bit and channels were split. Mean threshold was set for the neurite channel and region of interest (ROI) was measured for percentage of Area which was based on the positive signals compared to the ROI area measured. Additionally on the nucleus channel we set the threshold using Li thresholding and used the same ROI to measure the percentage Area. Subsequently Area measured in the nucleus channel was subtracted from the neurite channel in order to have the real percentage of area occupied by neurites. The data were then analysed using Graphpad prism 8.

LAMP1 and EEA1 volumes quantification. For these types of samples, we advised single-photon microscopy imaging. Were acquired with Laser scanning confocal microscope (A1R HD25, Nikon) this method provides high-quality imaging with high single-cell resolution. we used a Plan Fluor 60x oil objective. The images acquired with Resonant Scanner mode (1024 x 1024 pixels, scanning speed: 15 fps), activate the z-stack mode and define the lower and upper limits.

The cells were analysed using NIS Elements software 3.0 (Nikon). For 3D analysis we used 3 General Analysis (GA) option With Nis AI to analysis the sample. In this type of analysis, we used the artificial intelligent (AI) method for recovering contrast to improving signal to noise ratio. In the beginning, we removed automatically the shot noise component of images and the signal emitted from out-of-focus planes with Denoise.ai and Clarify.ai functions in Nis.ai. After we used the 3D segmentation and 3D Binary methods to detect and select each cell and identified EEA1

and LAMP1 staining in each cell. Subsequently, the volume, intensity of each vesicle per cell was quantified accordingly. The same parameters were used to quantify all the different images.

RNA extraction and sequencing. Total RNA was extracted using the RNeasy Plus Micro kit (Qiagen) according to the manufacturer's protocol. All RNAs were analyzed for quantity and quality. RNA concentrations were determined with High Sensitivity RNA ScreenTape on TapeStation (Agilent). cDNA libraries were prepared using KAPA mRNA Hyper Prep kit (Roche) according to the manufacturer's protocol. Sequencing was performed on a NextSeq 500 (Illumina) by the iGeneSeq ICM internal platform.

RNA-seq data analysis. Samples sequenced are : Blood monocytes derived macrophages (n=4 Ctrl), iPSC derived mono-cultured microglia (n=2 Ctrl), iPSC derived mono-cultured macrophages (n= 6 Ctrl, n=18 C9orf72^{(G4C2)n}, n=6 SOD1^{N139D}, n=3 SOD1^{H44R}, n=5 TARDBP^{G348C}), iPSC derived microglia co-cultured with matched MNs (n= 11 Ctrl, n= 13 C9orf72^{(G4C2)n}, n=4 SOD1^{N139D}, n=3 SOD1^{H44R}, n=5 TARDBP^{G348C}), iPSC derived macrophages co-cultured with matched MNs (n=4 Ctrl, n=12 C9orf72^{(G4C2)n}, n=2 SOD1^{N139D}, n=3 SOD1^{H44R}, n=3 TARDBP^{G348C}).

Quality of raw data was evaluated with FastQC. Poor quality sequences and adapters were trimmed or removed with fastp tool, with default parameters, to retain only good quality paired reads. Illumina DRAGEN bio-IT Platform (v3.8.4) was used for mapping on hg38 reference genome and quantification with gencode v32 annotation gtf file. Library orientation, library composition and coverage along transcripts were checked with Picard tools. Following analysis were conducted with R software. Data were normalized with limma (v3.48.3) bioconductor packages voom function prior to differential analysis. Multiple hypothesis adjusted p-values were calculated with the Benjamini-Hochberg procedure to control FDR. Finally, enrichment analysis was conducted with clusterProfiler R package (v3.14.3) with over-representation analysis and/or Gene Set Enrichment Analysis, on gene ontology database, REACTOME, KEGG, GO pathways.

Quantitative PCR. Reverse transcription of 1µg RNA into cDNA was performed with the SensiFAST cDNA synthesis kit (Bioline, France). Quantitative reverse transcription PCR was then performed in a Roche LightCycler480 using 100 ng of equivalent cDNA and the LightCycler

probes master according to Roche procedures. Primers were from ThermoFisher Scientific: Hs01881698_s1 and Hs02786624-g1 for P2RY12 and GAPDH, respectively.

Statistical analysis. All data are shown as mean \pm SEM and all analyses were conducted using Prism software (Graphpad Prism). Tests used are indicated in figure legends.

Lefebvre-Omar et al.* is a paper of the team that will be submitted before the end of 2021. A “almost ready” version of this paper is at the end of this manuscript. **“Neurofilament accumulations in motor neurons of patients with Amyotrophic lateral Sclerosis impair structural axonal initial segment plasticity”**. C. Lefebvre-Omar, E. Liu, C. Dalle, B. Lamotte d'Incamps, S. Bigou, C. Daube, Coline Jost-Mousseau, S. Blanchard, G. Tournaire, G. Bisch, C. Nicaise, M. Davenne, F. Salachas, L. Lacomblez, D. Seilhean, C. Lobsiger, S. Millecamps, S. Boillée, D. Bohl.

Liu et al* is a review in revision for publication in Frontiers in Molecular neurosciences. The document is at the end of this manuscript. **“Human Induced Pluripotent Stem cells to study neuroinflammation in ALS and FTD”**. Liu E., Karpf L., Bohl D.

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Conflict of interest statement

None

Figures and Supplementary Figures

Figure 1. Generation of iPSC-derived macrophages and microglia-like cells.

(a,b) Schematic representation of the protocols allowing the differentiation of iPSC into myeloid-like progenitors and their further differentiation either into macrophages or microglial-like cells with different media and cytokines. Flow cytometry on progenitor cells showed >95% of CD45+ progenitors (b left). Progenitors are matured in different media and specific culture condition for 7 or 14 days. Mature cells are negatively sorted and analysed by RNA sequencing. **(c)** DeSeq2 normalized gene expressions in blood-derived macrophages (MP), myeloid-like progenitors, iPSC-derived macrophages (MP) and microglial cells (MG) after negative cell sorting from co-cultures. **(d)** Upper: bright field images of iPSC-derived macrophages matured either 7 days alone in the presence of M-CSF (left image) or detached after these 7 days of culture and plated 7 more days onto MNs (right image). Scale bar: 50µm. Lower: confocal representative images of differentiated cultures immuno-labelled with antibodies against both CD45 and CD11b to stain macrophages (in green and in pink, respectively) with or without a β 3-tubulin antibody to stain neurons (in red). Nuclei were stained with Hoechst 33342 (Ho). Scale bar: 20µm. **(e)** Upper: bright field images of iPSC-derived microglia-like cells matured 14 days in the presence of IL-34, M-CSF, and TGF- β , alone or after plating of myeloid-like progenitors onto MNs. Scale bar: 50µm. Lower: confocal representative images of differentiated cultures immuno-labelled with antibodies against both CD45 and CD11b to stain microglia-like cells (in green and in pink, respectively), with or without a β 3-tubulin antibody to stain neurites (in red). Nuclei were stained with Hoechst 33342 (Ho). Scale bar: 20µm.

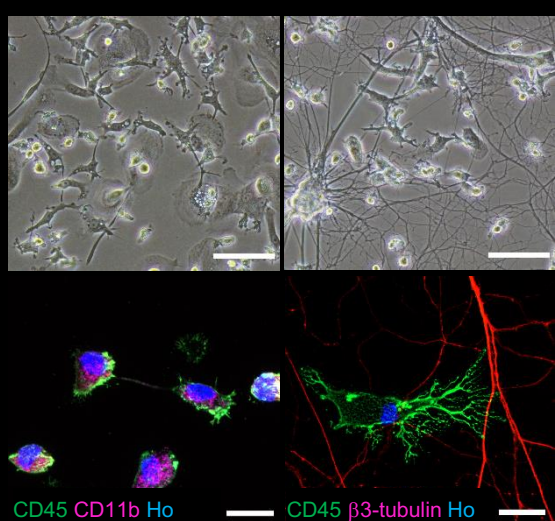
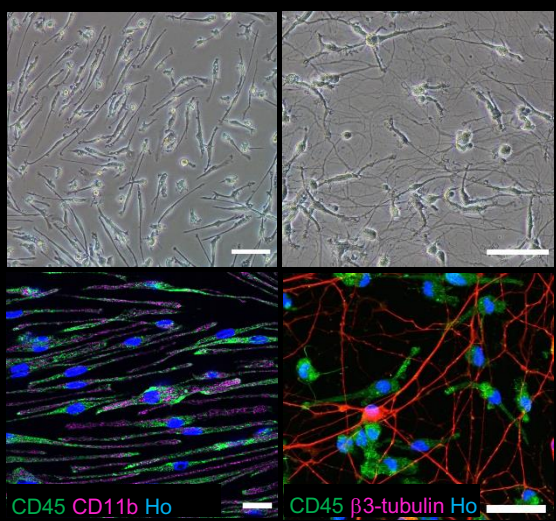
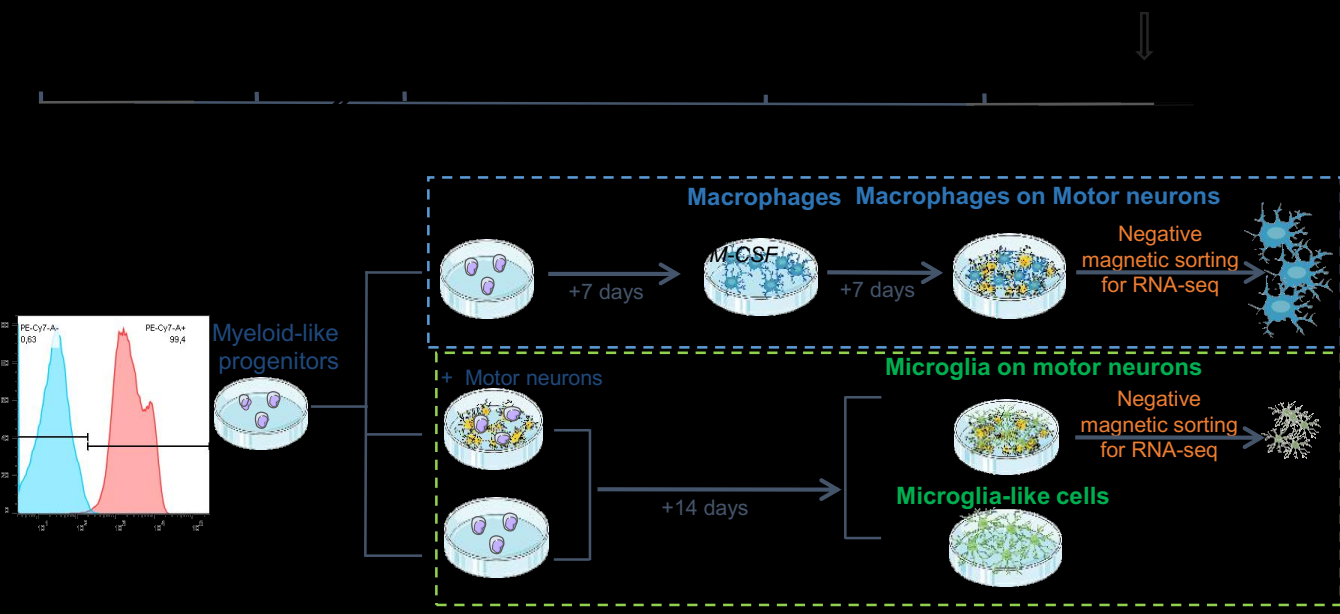


Figure 2. Transcriptome profiles of iPSC-derived macrophages and microglia-like cells.

(a) PCA of all expressed genes between control and mutant iPSC-differentiated cells, as well as blood-derived macrophages (bMP). PC1 vs PC2 is presented on the left. The first dimension of the PCA allow separation of monocultured macrophages (MP) and bMP from monocultured microglia (MG), and co-cultured microglia (MG_MN) and macrophages (MP_MN). PC1 vs PC3 is presented on the right. The third dimension allow discrimination between microglia and co-cultured microglia from co-cultured macrophages. No mutant specific clustering is observed.

(b) Heatmap showing expressions of consensus human macrophages and microglia markers in the different cell types mentioned in (a). Each column represents one cell sample (MG, MG_MN, bMP, MP, MP_MN) Colors indicate levels of expression of the gene with a maximum expression in red and a low expression in blue to white.

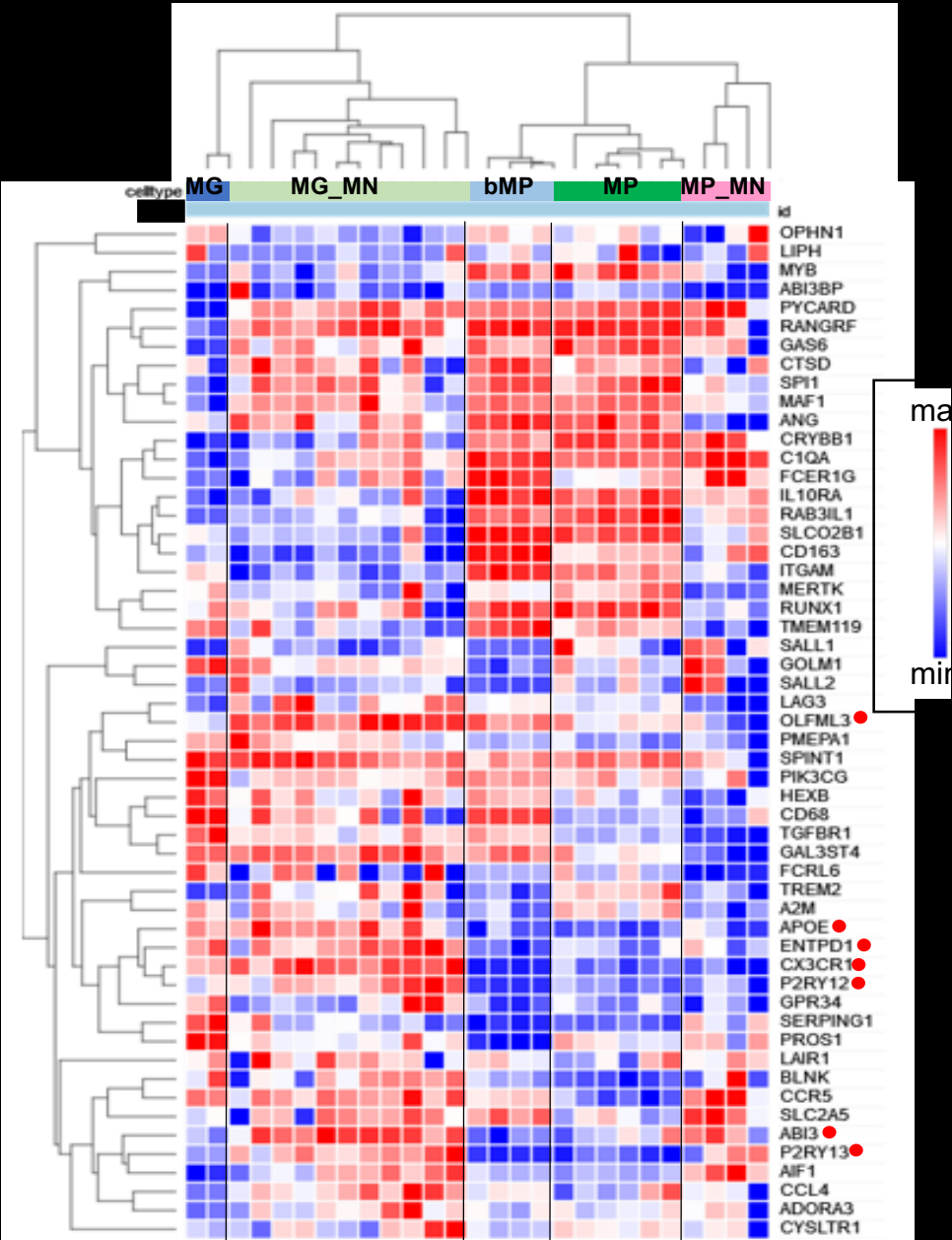
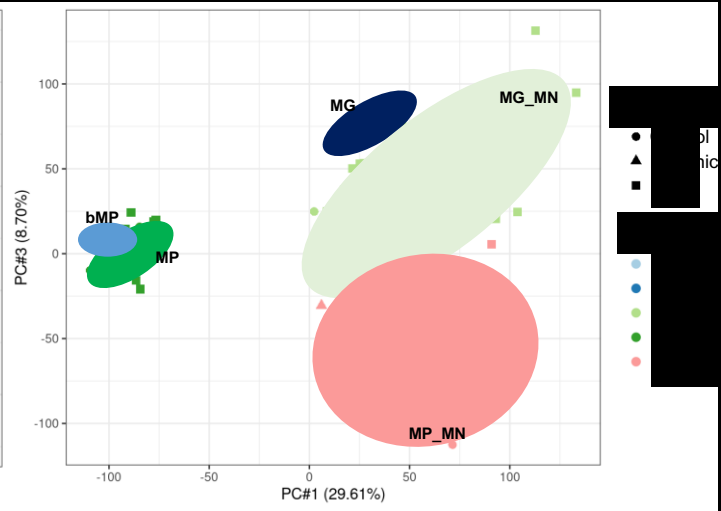
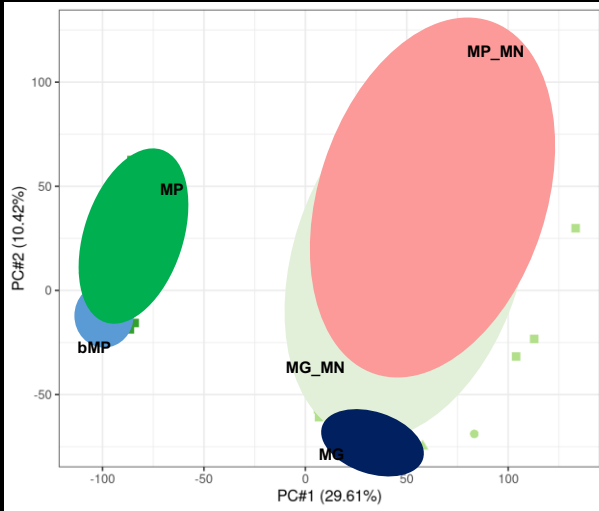
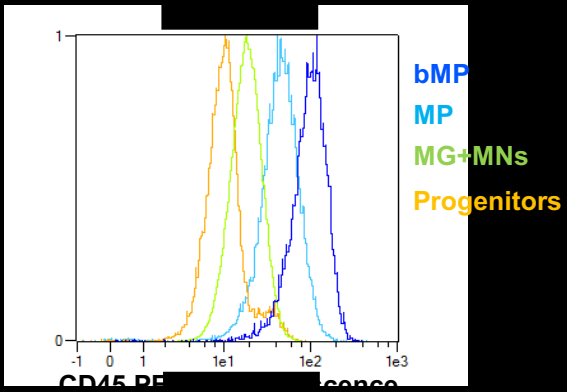


Figure 3. iPSC-derived macrophages and microglia are functional, can secrete cytokines and perform phagocytosis.

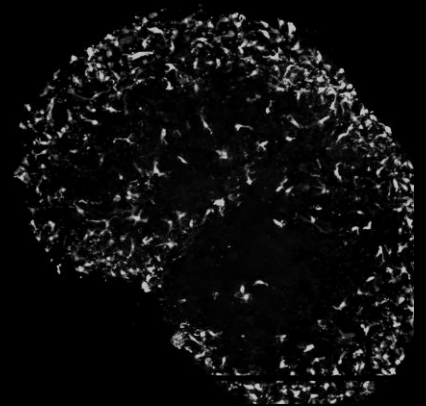
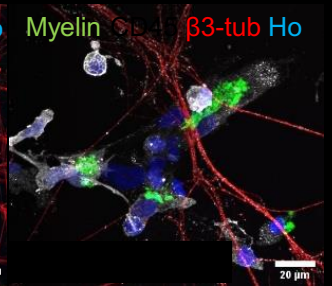
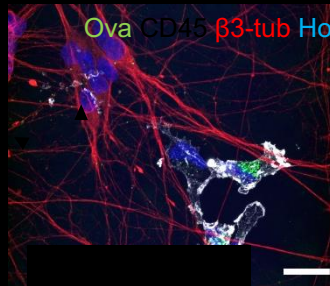
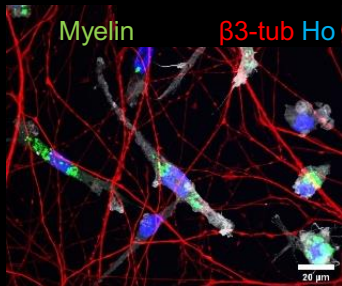
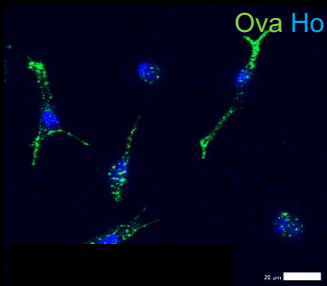
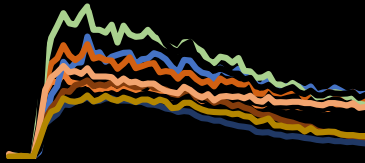
- (a) Flow cytometry analysis of CD45 expression on bMP, MP, MG_MN and progenitors.
- (b) P2RY12 mRNA expression levels measured by quantitative RT-PCR in macrophages and microglia-like cells matured on MNs. GAPDH = internal control for normalisation. (n = 6). Kruskal-Wallis test followed by Dunn's multiple comparisons test (***) $p < 0.001$).
- (c) Calcium imaging in cultures of iPSC-derived macrophages and microglia-like cells. Each line represents emission of fluorescence of one cell compared to background in response to ADP. Responses were recorded every second over two minutes.
- (d) Phagocytic capacities of ovalbumin-Alexa488 (Ova, in green) by macrophages cultured alone and of Cy3-tagged-myelin (in green) by macrophages (CD45, in white) cultured onto MNs (β 3-tubulin, in red). Cells were fixed after one hour of incubation. Macrophages and MNs were immuno-stained with antibodies directed against CD45 and β 3-tubulin, respectively. Nuclei were stained with Hoechst 33342 (Ho). Scale bar: 20 μ m
- (e) Phagocytic capacities of (left) ovalbumin-Alexa488 (in green) or of (right) Cy3-tagged-myelin (in green) by microglia-like cells matured onto MNs. Microglia and MNs were immuno-stained with antibodies directed against CD45 and β 3-tubulin, respectively. Nuclei were stained with Hoechst 33342 (Ho). Scale bar: 20 μ m
- (f) Concentrations of IL-10, IL-6 and IL-8 cytokines measured by ELISA in supernatants of iPSC-derived macrophages cultured alone or onto MNs, and of microglial cells matured onto MNs. (n=3 to 10 independent experiments).
- (g) Myeloid like progenitors injected into ventral APP mutant organoids of 2 months of age, cells were cultured for 7 days. They colonised the entire organoid (upper, scale bar: 500 μ m) and expressed specific markers (Iba.1, purple); they could also interact with cellular debris, here in Amyloid- β (green) (lower image) (scale bar: 10 μ m)



+ ADP



+ADP



Iba.1 Amyloid β>

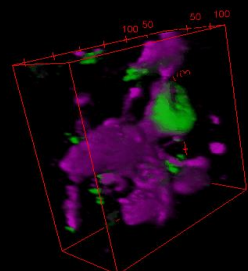


Figure 4. iPSC-derived MNs co-cultured with control and ALS macrophages.

(a) Representative confocal images of control (Ctrl) and ALS (C9orf72^{(G4C2)ⁿ}, SOD1^{N139D}, TARDBP^{G348C}) iPSC-derived MNs cultured 11 days in the medium dedicated to cultures with macrophages (MP). Neurite networks were immuno-stained with an antibody directed against β 3-tubulin (in white). Nuclei were stained with Hoescht H33342 (Ho, in blue). Scale bars: 50 μ m.

(b) Representative confocal images of matched co-cultures of control (Ctrl) and ALS (C9orf72^{(G4C2)ⁿ}, SOD1^{N139D}, TARDBP^{G348C}) MNs and macrophages (MP). Neurite networks were immuno-stained with an antibody directed against β 3-tubulin (in white) and macrophages were immune-stained with an antibody directed against CD45 (in green). Nuclei were stained with Hoescht H33342 (Ho, in blue). Scale bars: 50 μ m

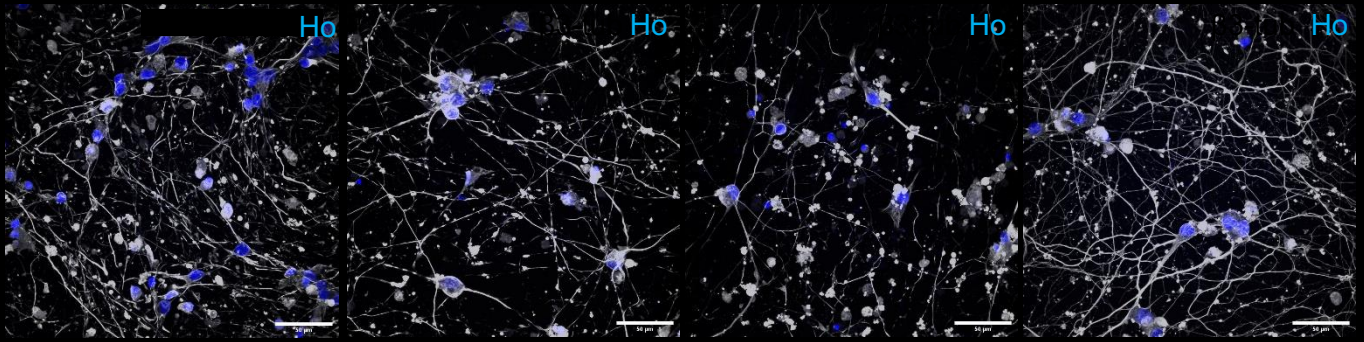
(c) Quantification of surfaces occupied by β 3-tubulin staining in the different cultures of MNs alone or in matched MN/MP co-cultures. (Mean +/- SEM) (n= 3 to 5 independent experiments).

(d) Quantification of surfaces labelled by β 3-tubulin staining in the different matched MN/MP co-cultures. Results show quantifications for the 4 different C9orf72^{(G4C2)ⁿ} patients (P1, P2, P3, P4), the SOD1^{N139D} patient (with 2 different clones analysed) and the TARDBP^{G348C} (with 3 different clones analysed) patient. (Mean +/- SEM) (n= 3 to 9 independent experiments). Statistical analysis: Kruskal-Wallis test followed by Dunn's multiple comparisons test (*p<0.05)

C9orf72 [G4C2]ⁿ

SOD1 N139D

TARDBP G348C



C9orf72 [G4C2]ⁿ
C9orf72 [G4C2]ⁿ

SOD1 N139D
SOD1 N139D

TARDBP G348C
TARDBP G348C

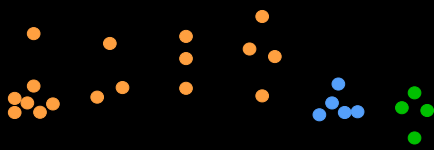
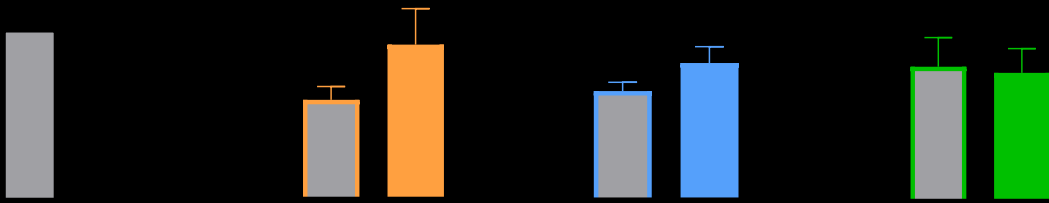
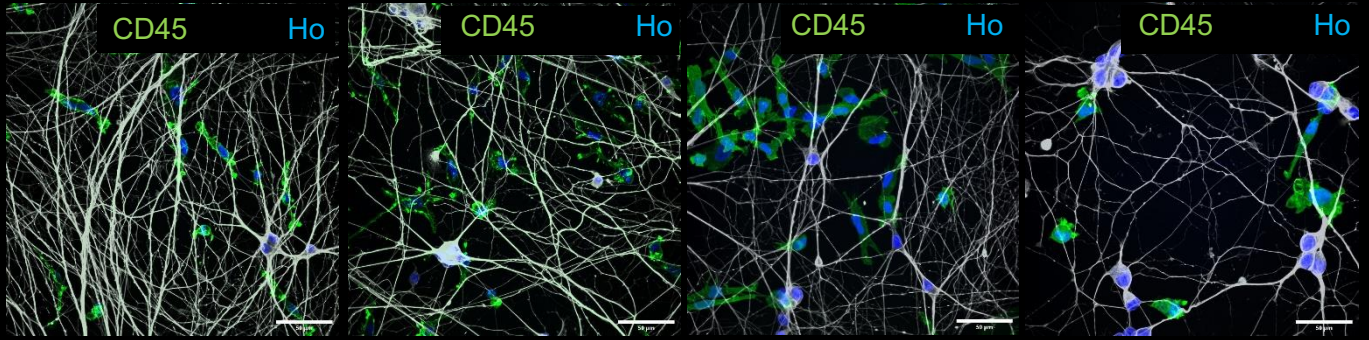


Figure 5. iPSC-derived MNs co-cultured with control and ALS microglia-like cells.

(a) Representative confocal images of control (Ctrl) and ALS (C9orf72^{(G4C2)ⁿ}, SOD1^{N139D}, TARDBP^{G348C}) iPSC-derived MNs cultured 14 days in the medium dedicated to co-cultures with iPSC-derived microglial-like cells (MG). Neurite networks were immuno-stained with an antibody directed against β 3-tubulin (in white). Nuclei were stained with Hoescht H33342 (Ho, in blue). Scale bars: 100 μ m

(b) Representative confocal images of matched co-cultures of control (Ctrl) or ALS (C9orf72^{(G4C2)ⁿ}, SOD1^{N139D}, TARDBP^{G348C}) MNs and iPSC-derived microglial-like cells (MG). Neurite networks were immuno-stained with an antibody directed against β 3-tubulin (in white) and MG were immuno-stained with an antibody directed against CD45 (in green). Nuclei were stained with Hoescht H33342 (Ho, in blue). Scale bars: 50 μ m.

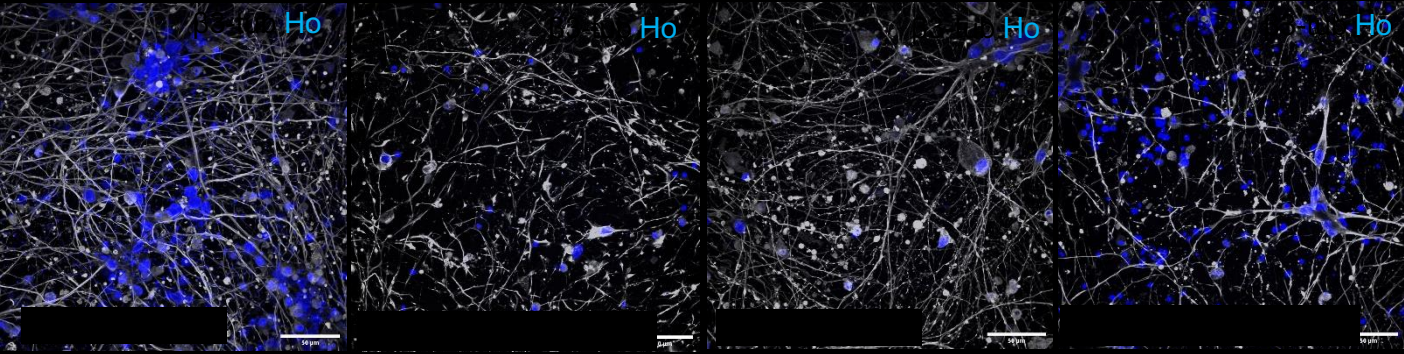
(c) Quantification of neurite network surfaces occupied by β 3-tubulin staining in the different cultures of MNs alone or in matched MN/MG co-cultures. (Mean +/- SEM) (n= 3 to 5 independent experiments).

(d) Quantification of neurite network surfaces labelled by β 3-tubulin staining in the different matched MN/MG co-cultures. Results show quantifications for the 4 different C9orf72^{(G4C2)ⁿ} patients (P1, P2, P3, P4), the SOD1^{N139D} patient (with 2 different clones analysed) and the TARDBP^{G348C} patient (with 3 different clones analysed). (Mean +/- SEM) (n= 3 to 6 independent experiments). Statistical analysis: Kruskal-Wallis test followed by Dunn's multiple comparisons test (*p<0.05)

C9orf72^{[G4C2]_n}

SOD1^{N139D}

TARDBP^{G348C}



C9orf72^{[G4C2]_n}
C9orf72^{[G4C2]_n}

SOD1^{N139D}
SOD1^{N139D}

TARDBP^{G348C}
TARDBP^{G348C}

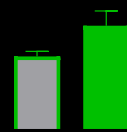
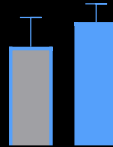
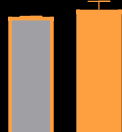
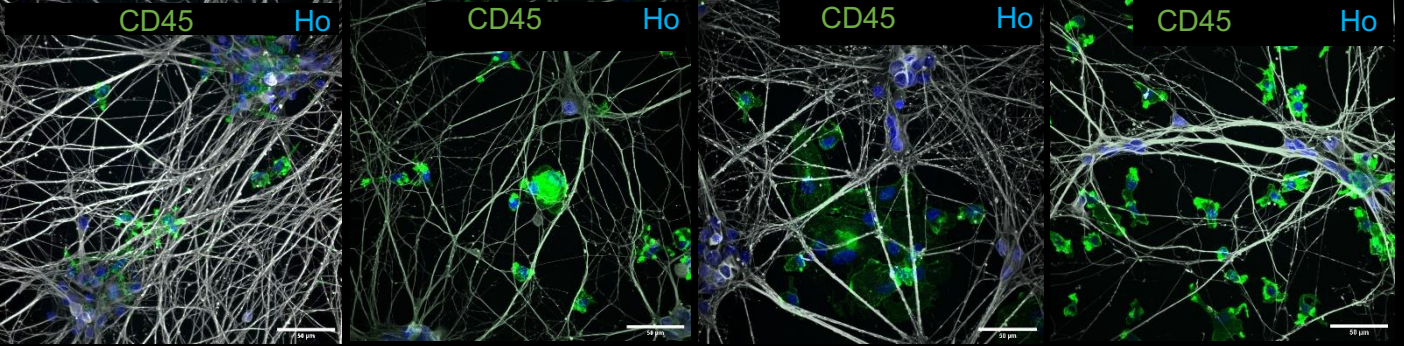


Figure 6. Alterations of the endolysosomal pathway in ALS macrophages.

(a) Representative confocal images of matched co-cultures between control or ALS (*C9orf72*^{(G4C2)ⁿ, *SOD1*^{N139D}, *TARDBP*^{G348C}) MNs and macrophages (MP) incubated with lysotracker (lyso, in red) and immuno-stained with antibodies directed against β 3-tubulin (in white) and CD45 (in green) (upper images, scale bar: 50 μ m; lower images, scale bar: 10 μ m). Nuclei were stained with Hoescht H33342 (Ho, in blue).}

(b) Representative confocal images of co-immuno-labelled co-cultures with LAMP1 (in green), EEA1 (in red) and β 3-tubulin (in white). Nuclei were stained with Hoescht H33342 (Ho, in blue). Scale bars: 10 μ m

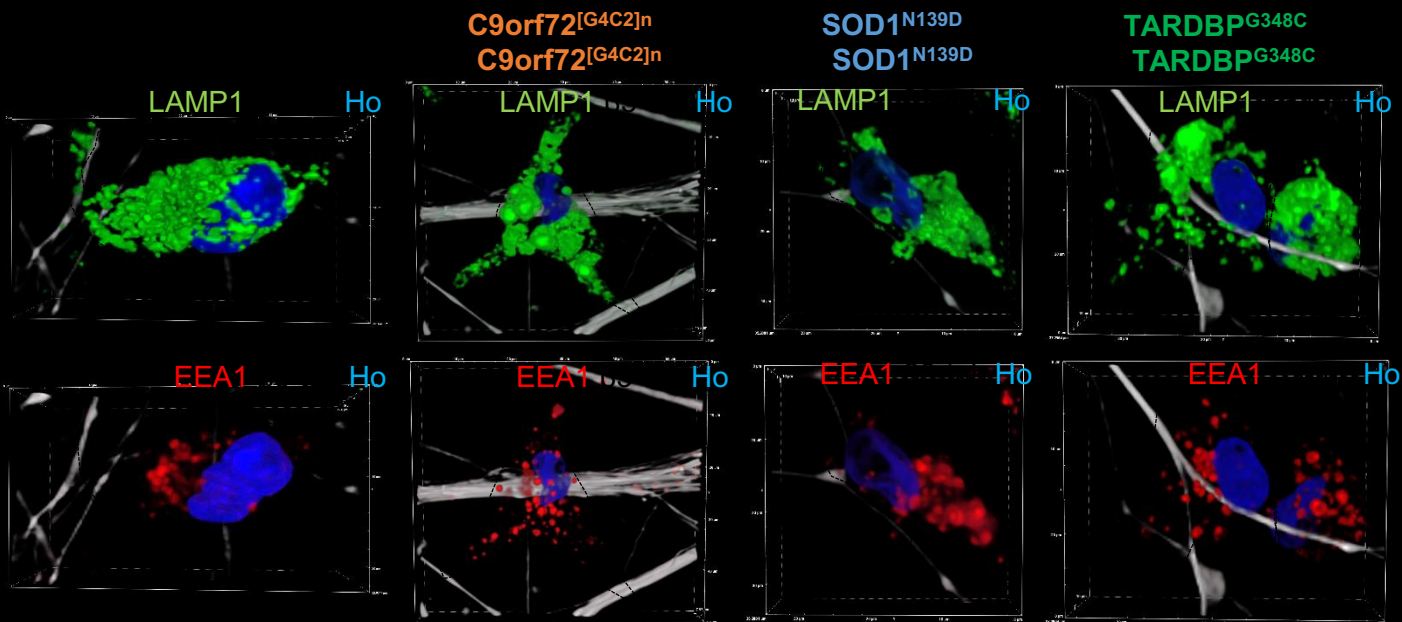
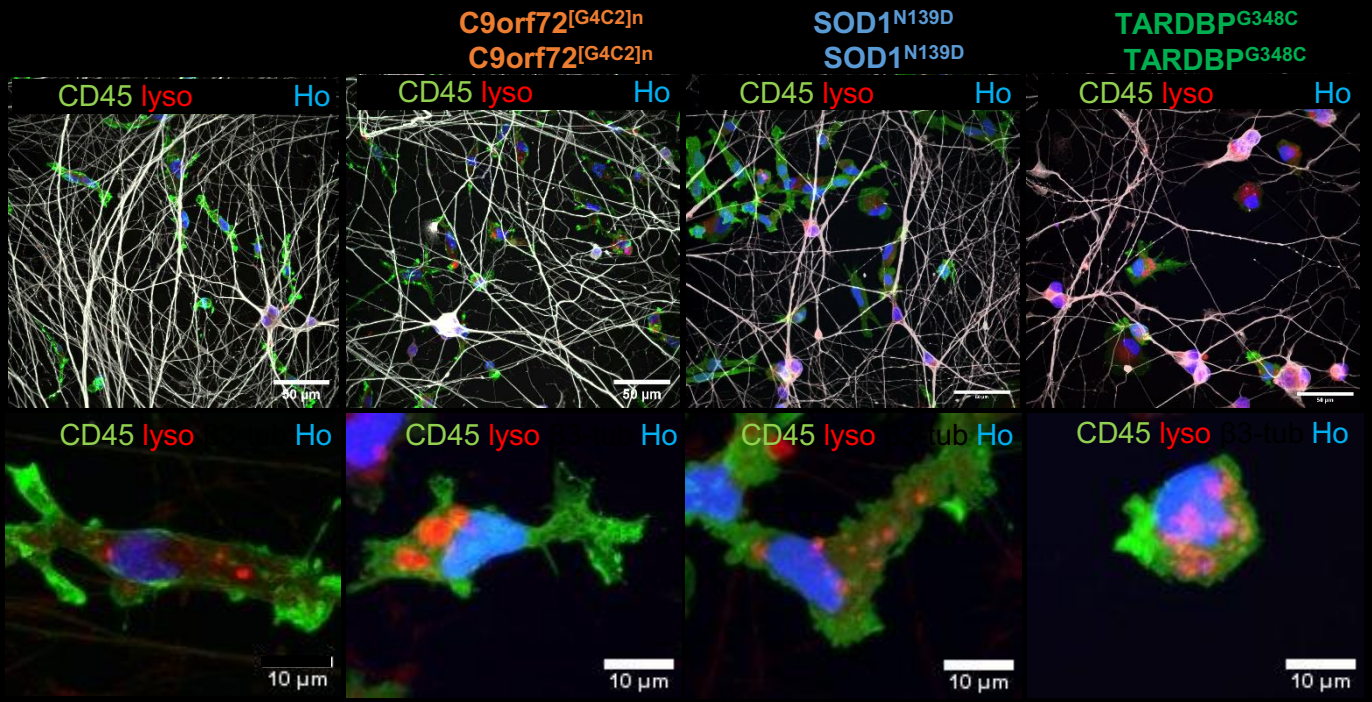


Figure 7. Quantifications of LAMP1 and EEA1 positive vesicles in ALS macrophages.

(a, c, e) (a) Number of LAMP1 positive vesicles per cell, (c) mean volumes (μm^3) of LAMP1 positive vesicles per cell, (e) all LAMP1 positive vesicles in all analysed cells (n=5447-26548 ves) in control and ALS macrophages in matched cultures (Mean \pm SEM). (n=3 to 5 independent experiments). Statistical analysis: Kruskal-Wallis test followed by Dunn's multiple comparisons test (*p<0.05, ***p<0.001, ****p<0.0001)

(b, d, g) (b) Number of EEA-1 positive vesicles per cell, (d) mean volumes (μm^3) of EEA-1 positive vesicles per cell, (g) all EEA-1 positive vesicles in all analysed cells (n=6390-29651 ves) in control and ALS macrophages in matched cultures. (mean \pm SEM). (n=3 to 5 independent experiments). Statistical analysis: Kruskal-Wallis test followed by Dunn's multiple comparisons test (*p<0.05, ***p<0.001, ****p<0.0001)

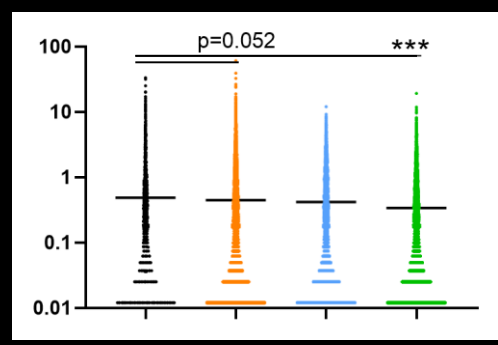
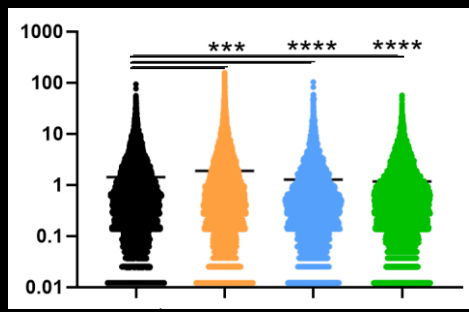
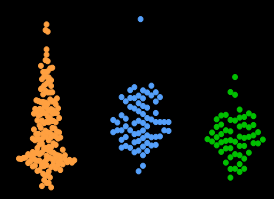
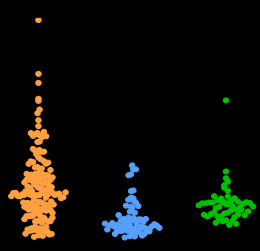
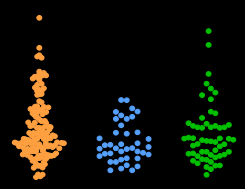
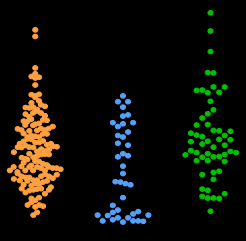


Figure 8. Alterations of the endolysosomal pathway in ALS microglia-like cells.

(a) Representative confocal images of matched co-cultures between control or ALS (*C9orf72*^{G4C2}_n, *SOD1*^{N139D}, *TARDBP*^{G348C}) MNs and microglia-like cells (MG) incubated with lysotracker (in red) and immuno-stained with antibodies directed against β 3-tubulin (in white) and CD45 (in green) (upper images, scale bar: 50 μ m; lower images, scale bar: 10 μ m). Nuclei were stained with Hoescht H33342 (Ho, in blue).

(b) Representative confocal images of co-immuno-labelled co-cultures with LAMP1 (in green), EEA1 (in red) and β 3-tubulin (in white). Nuclei were stained with Hoescht H33342 (Ho, in blue). Scale bars: 10 μ m

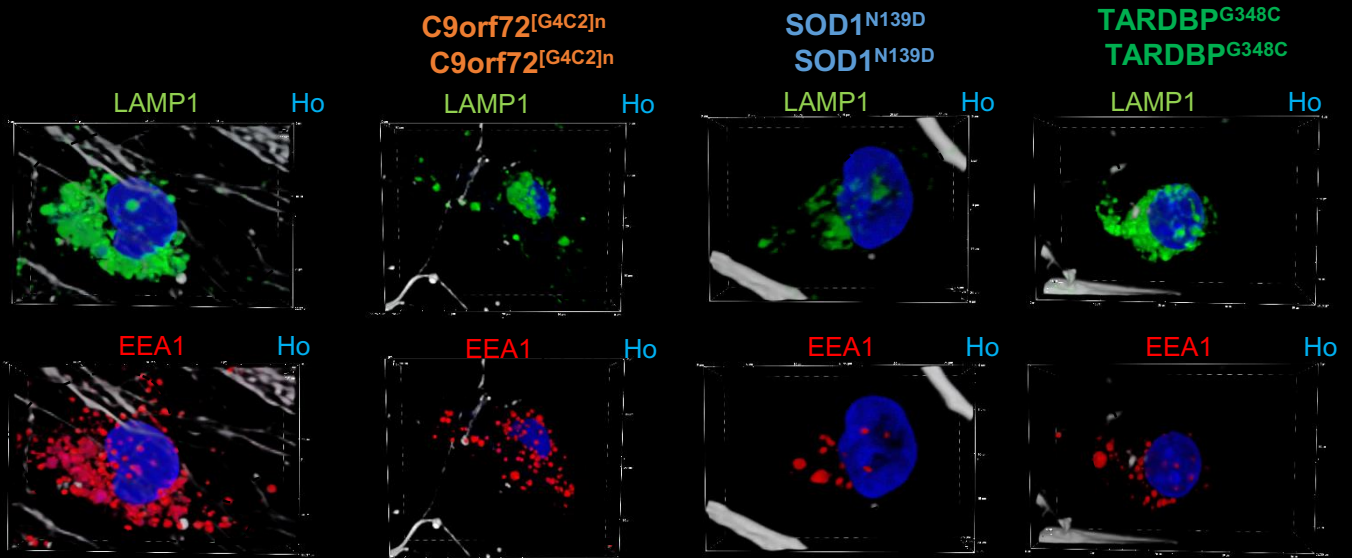
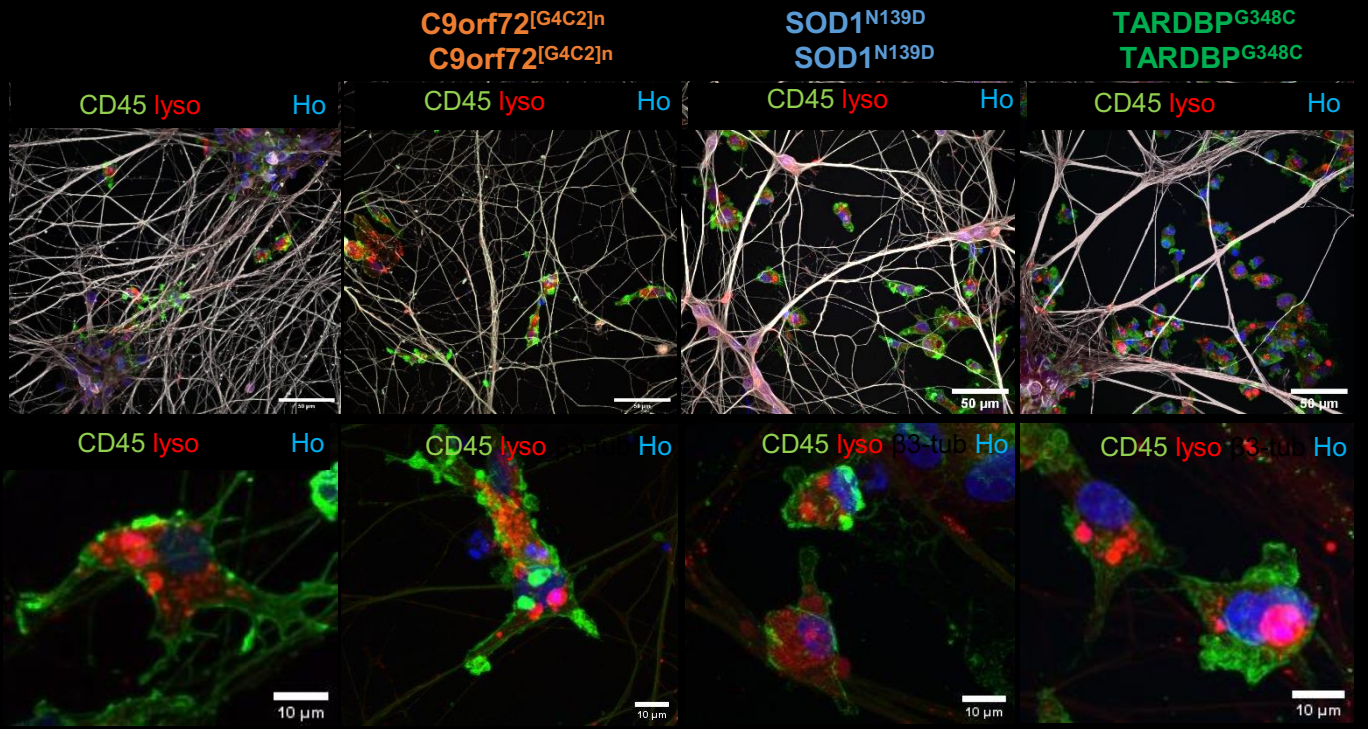


Figure 9. Quantifications of LAMP1 and EEA1 positive vesicles in ALS microglia-like cells.

(a, c, e) (a) Number of LAMP1 positive vesicles per cell, (c) mean volumes (μm^3) of LAMP1 positive vesicles per cell, (e) all LAMP1 positive vesicles in all analyzed cells ($n=900-9995$ ves) in control and ALS microglia-like cells in matched cultures. (mean \pm SEM). ($n=3$ to 5 independent experiments). Statistical analysis: Kruskal-Wallis test followed by Dunn's multiple comparisons test (* $p<0.05$, *** $p<0.001$, **** $p<0.0001$)

(b d, f) (b) Number of EEA-1 positive vesicles per cell, (d) mean volumes (μm^3) of EEA-1 positive vesicles per cell, (f) all EEA-1 positive vesicles in all analyzed cells ($n=4940-17052$ ves) in control and ALS microglia-like cells in matched cultures. (mean \pm SEM). ($n=3$ to 5 independent experiments). Statistical analysis: Kruskal-Wallis test followed by Dunn's multiple comparisons test (* $p<0.05$, *** $p<0.001$, **** $p<0.0001$)

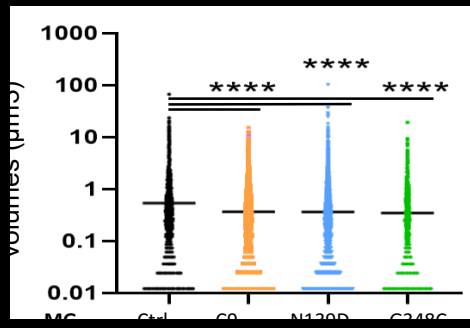
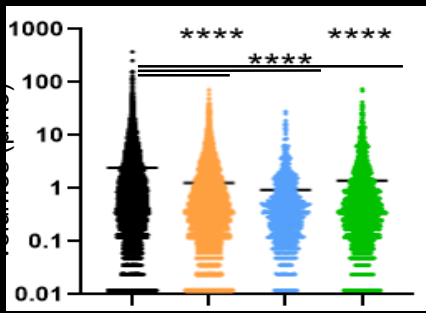
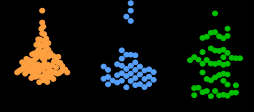
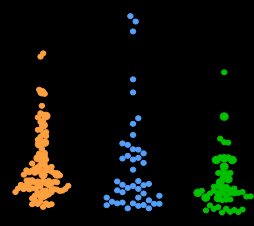
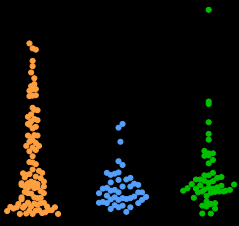


Figure 10. Graphs showing concentrations of the most deregulated inflammatory factors in ALS MNs and macrophages co-cultures compared to controls.

Graphs showing concentrations of (a) IL-10, (b) IL-8, (c) PAI-1, (d) PTX3, (e) sCD25, (f) sTNFR-I and (g) sTNFR-II measured in supernatants of co-cultures 3 days (3d) after the beginning of the co-culture and 11 days later (11d). Black dots correspond to co-cultures of control macrophages onto control MNs. Colored filled dots correspond to matched cultures between ALS ($C9orf72^{(G4C2)n}$, $SOD1^{N139D}$, $SOD1^{H44R}$, $TARDBP^{G348C}$) macrophages and MNs. For $C9orf72^{(G4C2)n}$ macrophages and $C9orf72^{(G4C2)n}$ MNs, each color represents one patient out of the four that were analysed. Colored empty dots correspond to mixed cultures of ALS macrophages ($C9orf72^{(G4C2)n}$, $SOD1^{N139D}$, $SOD1^{H44R}$, $TARDBP^{G348C}$) onto control MNs. Statistical analysis: two dimensional ANOVA analysis followed by Turkey's multiple comparison test (* $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$, **** $p < 0.00001$)

C9orf72^{[G4C2]_n}

SOD1^{N139D} SOD1^{H44R}

TARDBP^{G348C}

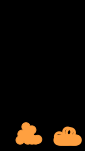
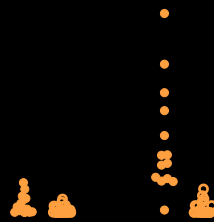
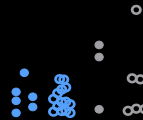
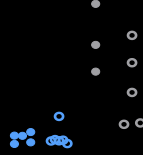
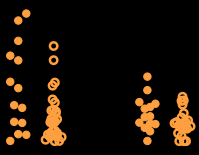


Figure 11. Graphs showing concentrations of the most deregulated inflammatory factors in ALS MNs and microglia-like cells co-cultures compared to controls.

Graphs showing concentrations of (a) IL-10, (b) IL-6, (c) IL-8, (d) TGF- β 1 (e) and PAI-1 measured in supernatants of co-cultures 3 days (3d) after the beginning of the co-culture and 11 days later (11d). Black dots correspond to co-cultures of control macrophages onto control MNs. Colored filled dots correspond to matched cultures between ALS (C9orf72^{(G4C2)_n}, SOD1^{N139D}, SOD1^{H44R}, TARDBP^{G348C}) microglia-like cells and MNs. Statistical analysis: two dimensional ANOVA analysis followed by Turkey's multiple comparison test (*p<0.05,**p<0.001, ***p<0.0001, ****p<0.00001).

C9orf72^{[G4C2]_n}

SOD1^{N139D} SOD1^{H44R}

TARDBP^{G348C}

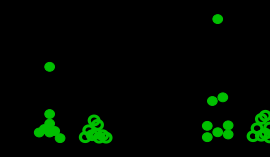
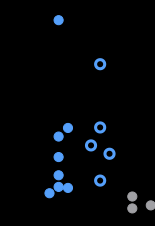
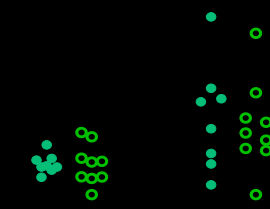
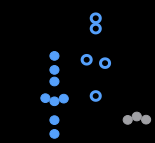
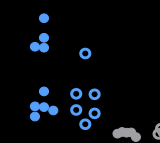
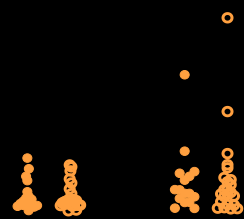
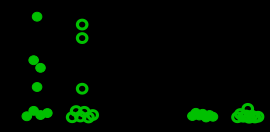
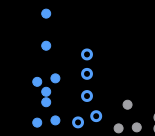
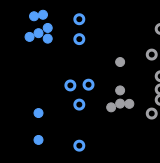
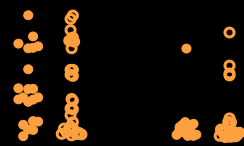
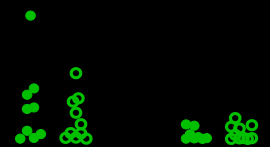
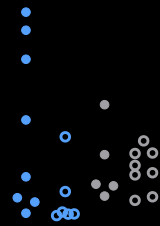
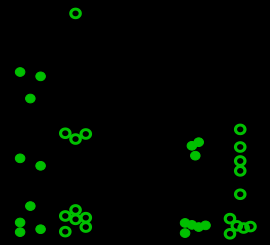
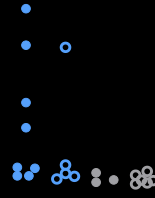
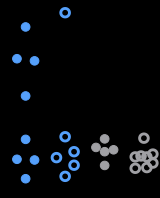
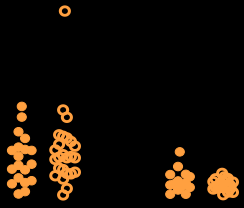


Figure 12. RNA sequencing analysis.

(a) Numbers of deregulated genes (up and down) in macrophages (MP) and microglia-like cells (MG) isolated from co-cultures with matched MNs. m= mutant.

(b, c, d) Dysregulated pathways in both macrophages and microglia-like cells isolated from matched co-cultures. m= mutant. Vs Ctrl means matched C9orf72, SOD1 or TARDBP co-cultures compared to matched control cultures.

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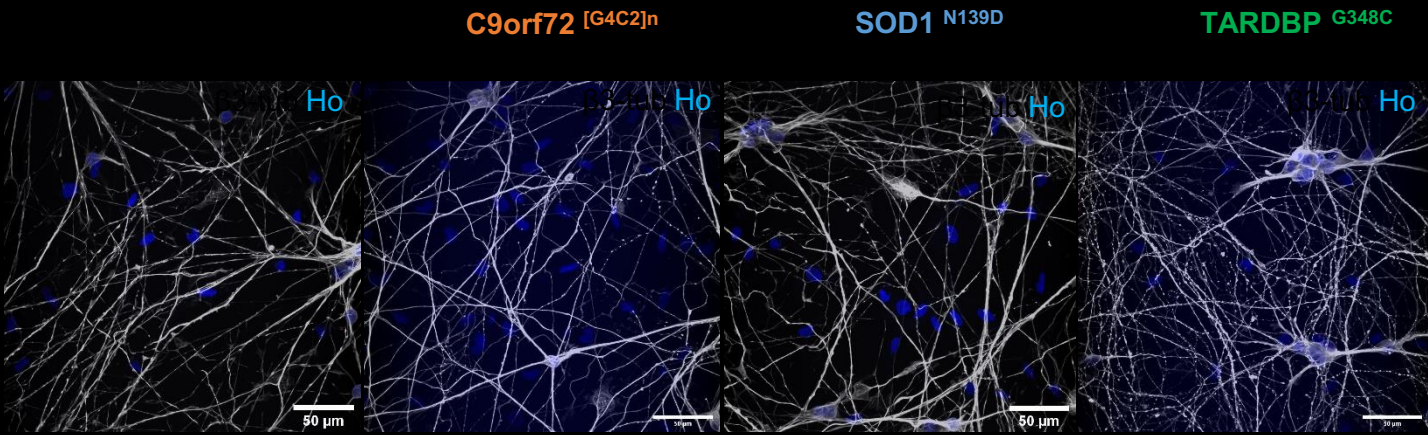
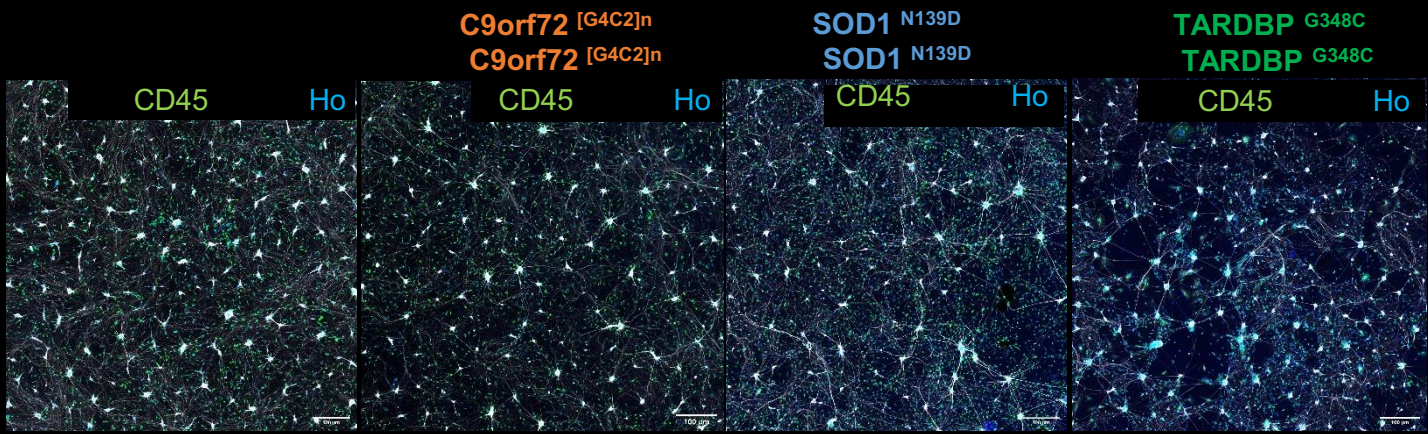
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Supplementary Figure 1. Complementary to Figure 4.

(a) Representative confocal images of matched co-cultures between control (Ctrl) or ALS ($C9orf72^{G4C2n}$, $SOD1^{N139D}$, $TARDBP^{G348C}$) MNs and macrophages (MP) immuno-stained with antibody directed against $\beta3$ -tubulin (in white) and against MP (CD45, green). Nuclei were stained with Hoescht H33342 (Ho, in blue). Scale bars: 100 μ m

(b) Representative confocal images of the control MNs neuritic network ($\beta3$ -tubulin, stained in white) after 11 days co-cultures with control (Ctrl) or ALS ($C9orf72^{G4C2n}$, $SOD1^{N139D}$, $TARDBP^{G348C}$) macrophages. Nuclei were stained with Hoescht H33342 (Ho, in blue). Scale bars: 50 μ m



Supplementary Figure 2. Complementary to Figure 5.

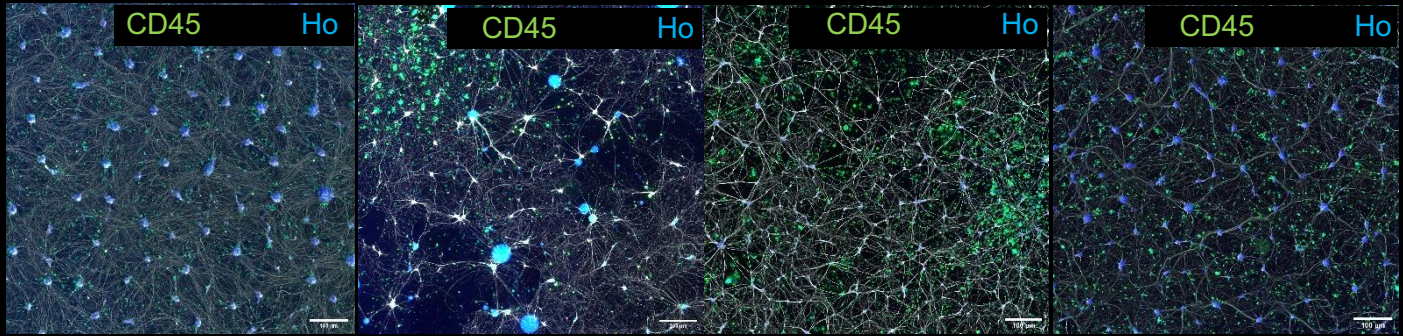
(a) Representative confocal images of matched co-cultures between control (Ctrl) or ALS ($C9orf72^{G4C2n}$, $SOD1^{N139D}$, $TARDBP^{G348C}$) MNs and microglia-like cells (MG) immuno-stained with antibody directed against $\beta3$ -tubulin (in white) and against MP (CD45, green). Nuclei were stained with Hoescht H33342 (Ho, in blue). Scale bars: $100\mu\text{m}$

(b) Representative confocal images of the control MNs neuritic network ($\beta3$ -tubulin, stained in white) after 14 days co-cultures with control (Ctrl) or ALS ($C9orf72^{G4C2n}$, $SOD1^{N139D}$, $TARDBP^{G348C}$) microglia-like cells. Nuclei were stained with Hoescht H33342 (Ho, in blue). Scale bars: $50\mu\text{m}$

C9orf72^{[G4C2]ⁿ}
C9orf72^{[G4C2]ⁿ}

SOD1^{N139D}
SOD1^{N139D}

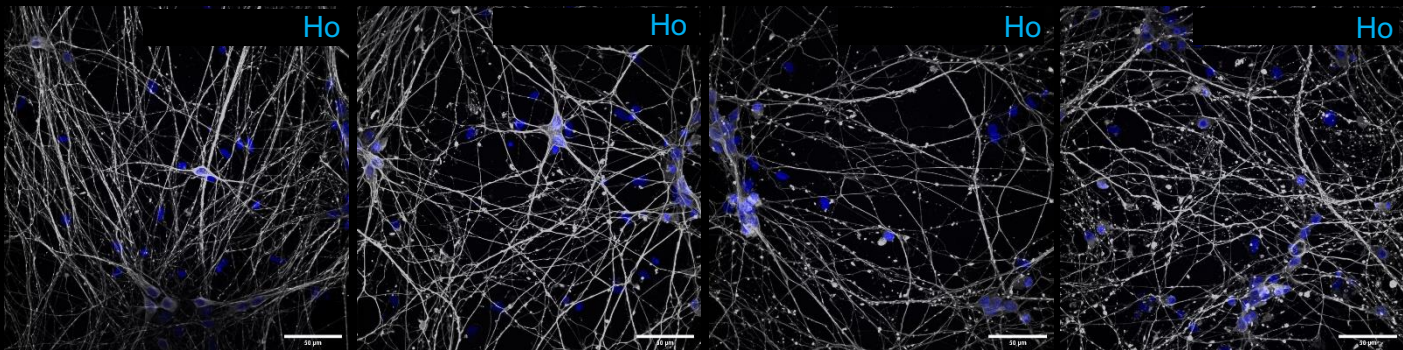
TARDBP^{G348C}
TARDBP^{G348C}



C9orf72^{[G4C2]ⁿ}

SOD1^{N139D}

TARDBP^{G348C}



Supplementary Figure 3. Complementary to Figure 6.

(a) Complementary to Figure 6a, lower images. Representative confocal images for each C9orf72 patient, a patient mutated in SOD1^{H44R} and its isogenic control (ISO-SOD1^{H44R}). Scale bar: 10 μ m.

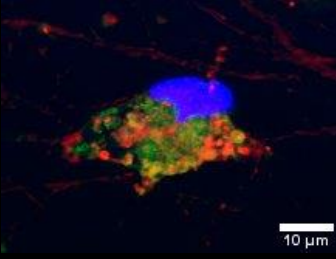
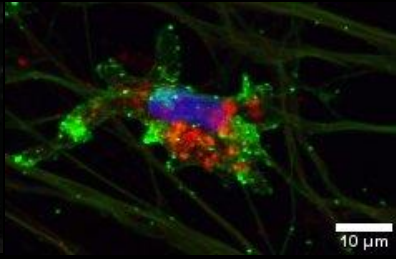
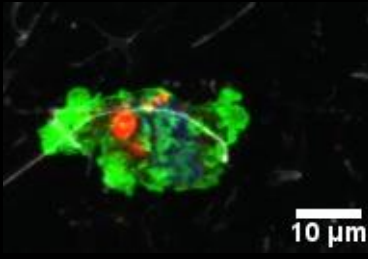
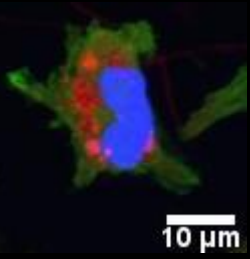
(b) Representative confocal images of co-cultures between control MNs and ALS (C9orf72^{(G4C2)ⁿ}, SOD1^{N139D}, TARDBP^{G348C}) macrophages (MP) incubated with lysotracker (in red) and immunostained with antibodies directed against β 3-tubulin (in white) and CD45 (in green) (upper images, scale bar: 50 μ m; lower images, scale bar: 10 μ m). Nuclei were stained with Hoescht H333342 (Ho, in blue).

C9orf72^{[G4C2]_n}

C9orf72^{[G4C2]_n}

Ho

CD45 lyso



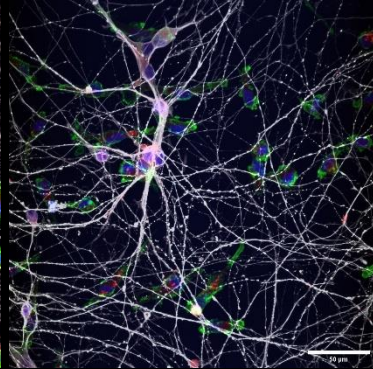
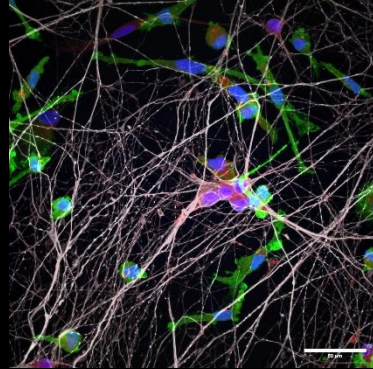
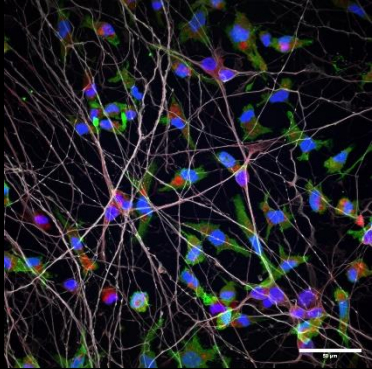
C9orf72^{[G4C2]_n}

SOD1^{N139D}

TARDBP^{G348C}

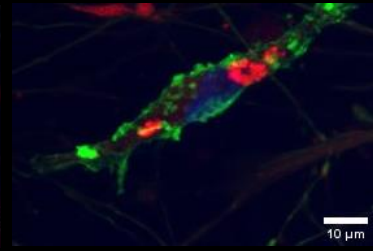
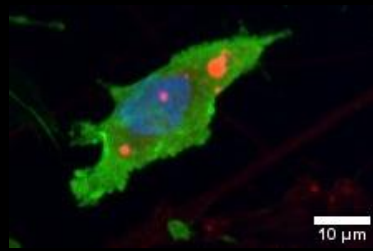
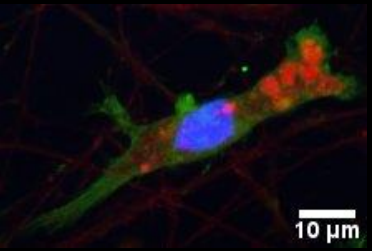
Ho

CD45 lyso



Ho

CD45 lyso



Supplementary Figure 4. MNs did not secrete or secrete at very low levels the 26 factors analysed in co-cultures. Graphs show concentrations (in pg/ml) of 26 inflammatory markers in supernatants of MNs cultured alone in the medium designed for co-cultures of MNs with macrophages. Supernatants were collected 3 days and 11 days (n = 2 to 4 samples analysed and shown as individual dots). In **(a)** are shown factors not detected in supernatants of co-cultures between MN and macrophages. In **(b)** are shown the 7 main deregulated factors secreted in ALS co-cultures between MNs and macrophages. In **(c)** are shown 5 factors found deregulated in ALS co-cultures between MNs and microglia-like cells.

Supplementary Figure 5. Same as Supplementary figure 4 for MNs cultured alone in the medium dedicated to co-cultures of MNs with microglia-like cells. In **(a)** are shown factors not detected in supernatants of co-cultures between MN and microglia-like cells. In **(b)** are shown the 7 main deregulated factors secreted in ALS co-cultures between MNs and microglia-like cells. In **(c)** are shown 3 factors found deregulated in ALS co-cultures between MNs and macrophages.

Supplementary Figure 6. Complementary to Figure 10.

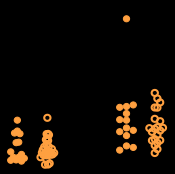
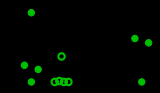
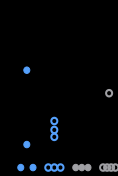
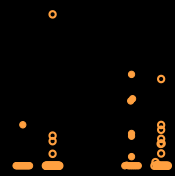
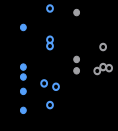
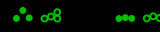
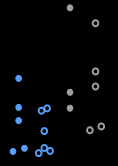
(a) Graphs showing concentrations (in pg/ml) of IL-6, MCP-1, sCD40L and sCD130 in co-cultures of MNs and macrophages. (b) Graphs showing concentrations (in pg/ml) of sCD25, sTNFR-I and sTNFR-II in co-cultures of MNs and macrophages. Black dots correspond to co-cultures of control macrophages onto control MNs. Colored filled dots correspond to matched cultures between ALS (C9orf72^{(G4C2)_n}, SOD1^{N139D}, SOD1^{H44R}, TARDBP^{G348C}) macrophages and MNs. Statistical analysis: two dimensional ANOVA analysis followed by Turkey's multiple comparison test (*p<0.05, **p<0.001, ***p<0.0001, ****p<0.00001)

C9orf72[G4C2]_n

SOD1^{N139D}

SOD1^{H44R}

TARDBP^{G348C}

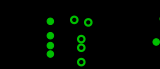
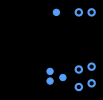
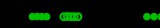
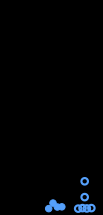
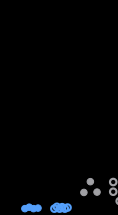


C9orf72[G4C2]_n

SOD1^{N139D}

SOD1^{H44R}

TARDBP^{G348C}



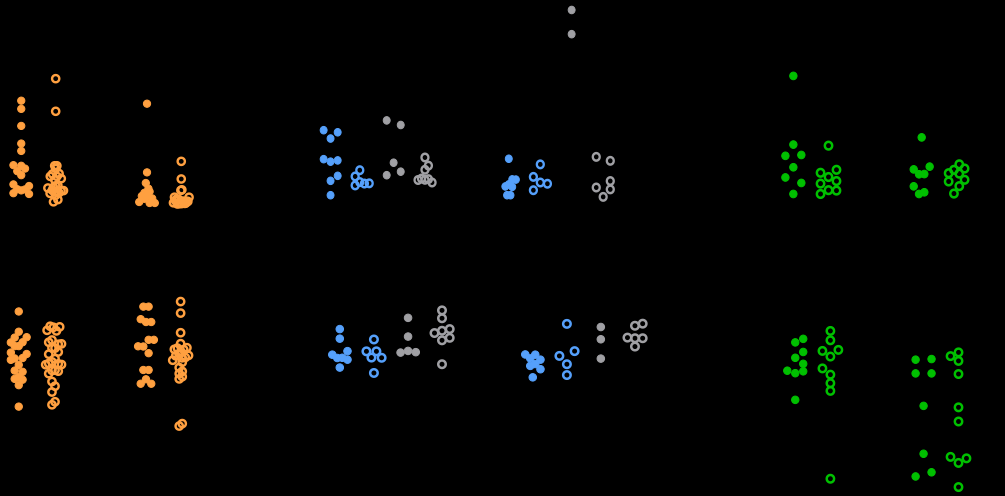
Supplementary Figure 7. Complementary to Figure 11.

(a) Graphs showing concentrations (in pg/ml) of IPTX3 and MCP-1 in co-cultures of MNs and microglia-like cells. (b) Graphs showing concentrations (in pg/ml) of sTNFR-I, sTNFR-II and sCD130 in co-cultures of MNs and microglia-like cells. Black dots correspond to co-cultures of control macrophages onto control MNs. Colored filled dots correspond to matched cultures between ALS (C9orf72^{(G4C2)ⁿ}, SOD1^{N139D}, SOD1^{H44R}, TARDBP^{G348C}) microglia-like cells and MNs. Statistical analysis: two dimensional ANOVA analysis followed by Turkey's multiple comparison test (*p<0.05, **p<0.001, ***p<0.0001, ****p<0.00001)

C9orf72^{[G4C2]_n}

SOD1^{N139D} SOD1^{H44R}

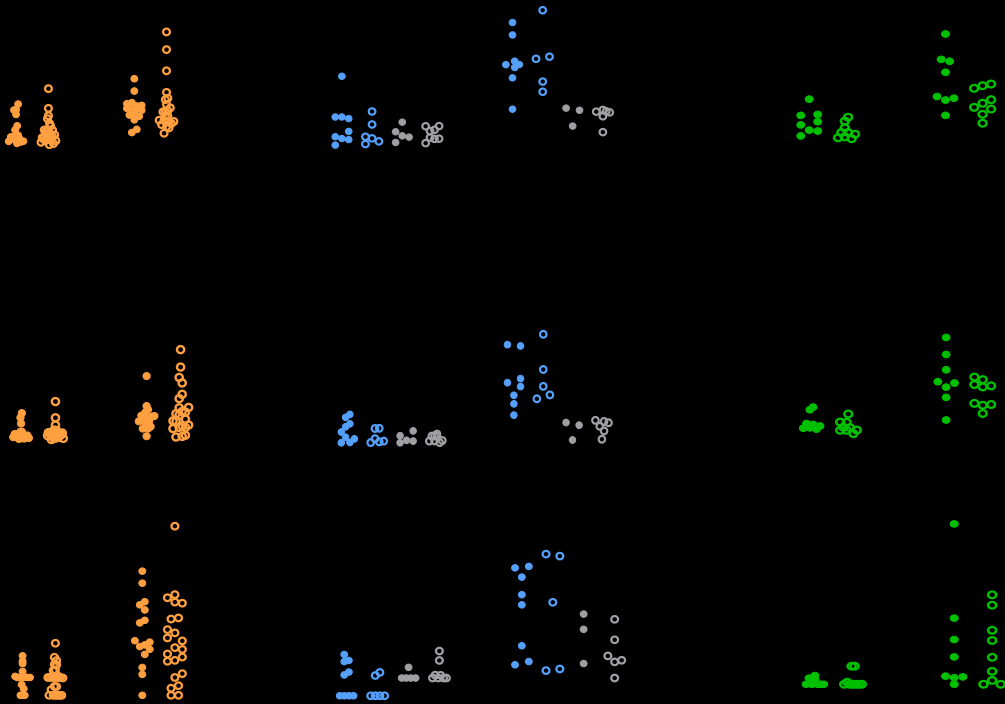
TARDBP^{G348C}



C9orf72^{[G4C2]_n}

SOD1^{N139D} SOD1^{H44R}

TARDBP^{G348C}



Supplementary video 1. Time-lapse imaging of matched co-cultures of macrophages and MNs (control, C9orf72^{(G4C2)_n} , SOD1^{N139D} , TARDBP^{G348C}) incubated with lysotracker. Altered intracellular dynamics of vesicles are obvious in ALS macrophages vs control ones.

Supplementary Table 1. ALS patients, control subjects and iPSC clones.

| | | | | | |
|------------------------------|-------|-------|-----------------|---------------|--|
| SOD ^{N139D} -1 | | | | | |
| SOD1 ^{N139D} -2 | woman | 50 | Lower limbs | >25 years | |
| SOD1 ^{N139D} -3 | | | | | |
| SOD1 ^{N139D} -ISO | | | | | |
| SOD1 ^{H44R} -1 | woman | 65 | Pre-symptomatic | Not described | |
| SOD1 ^{H44R} -ISO | | | | | |
| ¹ C9ORF72-1 | man | 70 | Lower limbs | 3 years | |
| ¹ C9ORF72-2 | | | | | |
| ² C9ORF72-1 | | 51 | | | |
| ² C9ORF72-2 | man | | Bulbar | 3 years | |
| ² C9ORF72-3 | | | | | |
| ³ C9ORF72 | man | 45 | Not described | 4 years | |
| ⁴ C9ORF72 | man | 46 | Upper limbs | Not described | |
| TARDBP ^{G348C} -1 | | | | | |
| TARDBP ^{G348C} -2 | man | 53 | Lower limbs | 6 years | |
| TARDBP ^{G348C} -3 | | | | | |
| TARDBP ^{G348C} -ISO | | | | | |
| Ctrl69-1 | woman | 69 | - | - | |
| Ctrl64 | man | 64 | - | - | |
| Ctrl40 | man | 40-45 | - | - | |
| CtrlSLi | man | 45-49 | - | - | |
| Ctrl Kute | woman | 25-29 | - | - | |

ISO = isogenic control iPSC clone generated with the CRISPR/CAS9 technology.

Other results

In collaboration with the team of Dr. Peyrin, we have developed a new model of microfluidic devices which permit us to strictly separate soma from axonal compartment while preventing innate immune cell trans-migration.

When we first started, as our aim was to compare to role of macrophages and microglia on MNs survival we sought to have a model allowing us to study either their respective contribution but also their synergic contribution, for that we had to ensure that cells were not passing from one compartment to the other. We first used a model of microfluidic chips, which had straight grooves, which had narrower ending in order to guide the axon towards the other side of the chamber. In these experimental conditions, we have tested two sizes of grooves at the narrowest end one of 3 μ m and the other one at 5 μ m (**Figure 1a, 1b**). **Figure a** shows clearly that MNs axons were suffering when the micro grooves of 3 μ m were used. Thus, we have used only 5 μ m grooves for subsequent development. The fragmentation observed was mainly because human MNs had larger axons compared to primary culture mice MNs.

However, we noticed that the use of 5 μ m chamber leads to important trans-migration from microglia but mostly from macrophages (**Figure 1c,d**). In fact, when microglia were added in the left chamber we observed that some of them were migrating and staying in the grooves, they did not tend to migrate importantly in the other side chamber (Figure 1b). However, macrophages which were plated in the right chamber displayed important trans-migration towards the cell soma chamber (**Figure 1d**). This defect had to be solved before moving forwards.

Dr. Peyrin and Dr. Courte previously developed a model based on “return to sender” strategy, by creating asymmetrical grooves which orientate the axon back to its supposed compartment (Courte et al. 2018). We have worked on several prototypes of this modified version before designing a model, which allowed neuronal survival, oxygen concentration sufficiency, and prevented cells trans migration.

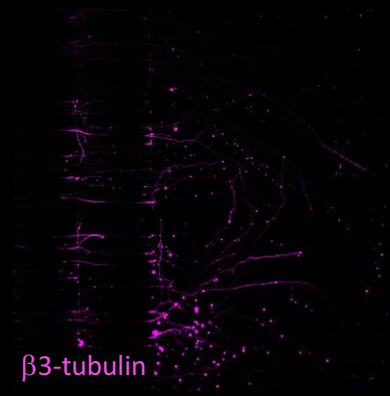
In parallel with the trans-migration phenomenon, we observed adherence cycling of macrophages and microglia, which were detached or loss over time due to the import flow generated between the two “media-changing-holes” each time the media was changed. In order to prevent this

phenomenon, bottleneck format was adapted at the entrance of the chamber in order to slow down the flow and prevent mechanical stress and detachment of innate immune cells upon media changing (**Figure 1e**).

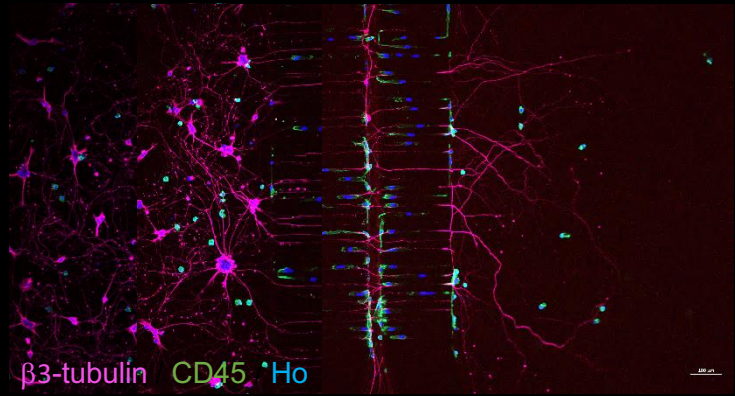
Taken together, we have implemented these different parameters onto a new chip (Figure 2). We have seeded macrophages and microglia in one or the other chamber and observed no migration over time supporting the functionality of the prototype we have designed. The next step will be to validate our previous observations made on coverslips in these models, by first realising co-cultures with iMP in the axonal compartment and iMG in the soma compartment. With the perspective to ultimately realise tri-cultures with iMG in the soma compartment and iMP in the axonal compartment, in order to unravel the synergic effects of the two cell types on MNs survival.

Figures

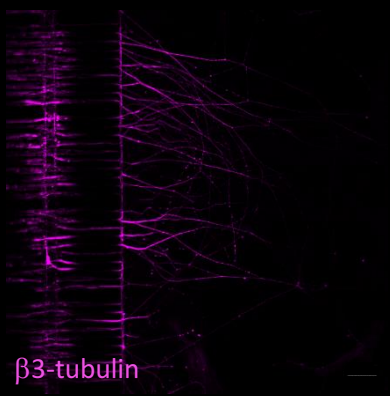
Figure 1 of other results. Development of new microfluidic chips to study macrophages and microglial cells into separate chambers. (a, b). MN precursors were plated in the left chambers of chips with 3 μ m or 5 μ m microgrooves. One week later, cultures were immuno-stained with an antibody against β 3-tubulin. (c, d) In chips with 5 μ m microgrooves, myeloid-like progenitors were plated and differentiated. In (c) progenitors were plated only in the left chamber in contact with soma of MNs. Two weeks later, cultures were immuno-stained with antibodies against β 3-tubulin and CD45 to label iMicroglia. Nuclei were stained with Hoescht 33243 (Ho). Scale bar: 100 μ m . In (d) progenitors were plated only in the right chamber in contact with axons of MNs. Two weeks later, same immuno-labellings were done on cultures as in (c). (e) bright-field images of progenitors were in two different chips were different holes. When using the chip shown on the right, cells remained attached and were not eliminated at each medium change. Scale bar: 500 μ m



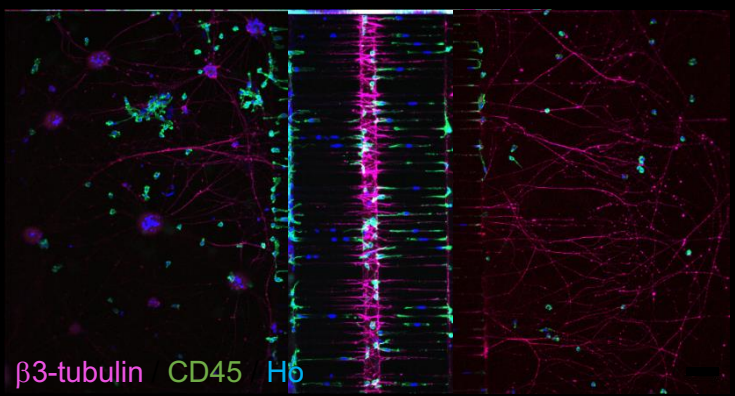
$\beta 3$ -tubulin



$\beta 3$ -tubulin CD45 Ho



$\beta 3$ -tubulin



$\beta 3$ -tubulin CD45 Ho

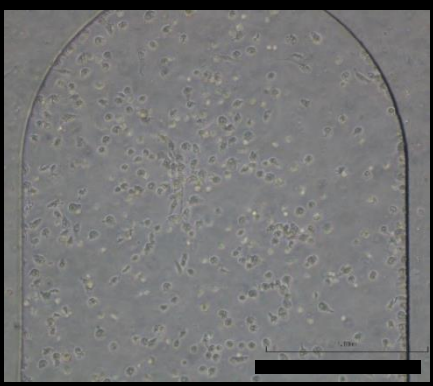
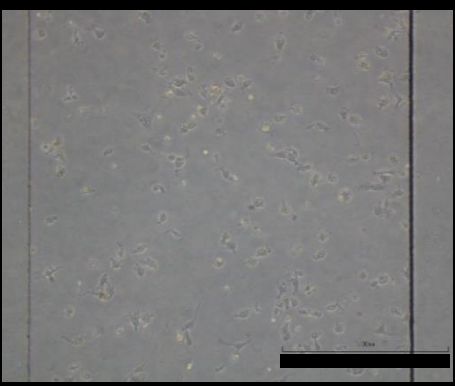


Figure 2 of other results. Immunostaining of new developed chips. Same protocol for cell culture and staining as in Figure 1. Scale bars: 500µm.

DISCUSSIONS & PERSPECTIVES

ALS disease is a fast fatal progressive disease, which affects a substantial number of person per year. To date there no cure for ALS. Current treatments are rather treatment options based on symptoms management and respiratory support as only modest benefits are observed and are not efficient for all ALS patients. One interesting avenue is gene therapy, which is beginning to emerge and offers new possibilities for some forms of genetic patients. However, this is not an option for the vast majority of patients with no identified genetic cause.

Regarding the pathomechanisms underlying the disease, many tracks are being considered and interesting hypotheses are flourishing. Mutant SOD1 mouse models have been widely used until today to portray an integrative picture of the different components of the pathology. In fact, thanks to this model, it was possible to correlate the direct involvement of innate immune cells and the progression of the pathology. This has opened up new hypotheses about the broader involvement of the immune system in the disease. These observations were supported by evidence of the presence of inflammation in the vast majority of cases of fALS and sALS. Not only at the level of post-mortem studies but also during the course of the disease thanks to the imaging studies, biofluids assessments, and also more recently to the transcriptomic studies carried out on the peripheral circulating cells of ALS patients. However, whether these factors have an active role in the pathology or are consequences of the neurodegeneration, remains enigmatic. The cause and effect link have only been demonstrated in mutant SOD1 mouse models, which do not necessarily reflect the entirety of the SLA subtypes. Thus, there is a consequent lack of understanding of the role of innate immune cells in all ALS forms. In this context, the use of iPSC represents an interesting tool to attempt to describe and explore the involvement of these innate immune cells using human material and in different genetic backgrounds, while keeping some limitations.

1- Tools developed to study inflammation in ALS

1.1 Human induced pluripotent stem cells

The technology of generation of human iPSC is particularly interesting to study ND, as it allows the study of human cells with their own genetic background, including sporadic cases. It allows also to generate a myriad of different cell types including those that are not accessible in alive patients, such as MNs and microglia.

In the laboratory, we dispose of 9 ALS iPSC cell lines mutated in different ALS genes as well as 2 sporadic ALS patients' derived iPSC lines. Importantly, for some of the iPSC lines we already have isogenic controls and the iPSC ICM platform is currently generating some other isogenic controls – especially for C9orf72 expansions – in which it will be interesting to validate our observations. This will let us to better understand the contribution of the mutation in the phenotypes we have observed. In my work I have focussed and compared in similar experimental conditions four C9orf72, two SOD1 and one TARDBP mutated patients, making a proof of concept. Further studies using sporadic cases as well as other fALS iPSC would be now interesting in order to observe commonalities and divergences between the ALS subtypes and try to identify sub-groups of phenotypes amongst ALS cases.

Although iPSC cultures remain an in vitro model bearing its limitations, in the past year several clinical trials have emerged from studies of iPSC derived MNs (Imamura et al. 2017; 2021; Okano et al. 2020). This support the use of iPSC to study human disease cells as they can recapitulate some of the in vivo pathological signatures.

1.2 Protocols to generate iPSC derived innate immune cells

To date several protocols have been established to generate iPSC-derived macrophages (iMP) and microglia (iMG). Most of them have attempted to mimic embryonic hematopoietic steps in order to obtain myeloid progenitors, which were then matured in microglial cells and peripheral macrophages using specific factors.

In the laboratory, we aimed to study microglia and peripheral tissue resident macrophages roles in ALS disease. When I began my PhD thesis, no protocol permitted the generation of both subtypes of macrophages using a unique protocol. Based on a published protocol we developed our own protocol allowing generation of both cell types expressing specific cell types signatures (Yanagimachi et al. 2013; Douvaras et al. 2017).

1.2.1 The challenge in obtaining a common progenitor that could be differentiated in both immune innate cell types

In the literature, it has been shown that microglia are derived from Myb-independent, Runx1, PU.1 Irf8 dependent progenitors (Kierdorf et al. 2013). Regarding the ontogeny of tissue-resident macrophages, the subject is still debated. The model proposed by Gomez-Perdiguero et al. suggests that most of the adult peripheral tissue-resident macrophages are derived from YS progenitors, which are similar to those colonising the brain early on (Gomez Perdiguero et al. 2015). Based on the understanding of microglia and tissue-resident macrophages ontogeny, we have adapted a previously published protocol from Yanagimachi et al. to obtain a macrophage and microglia common iPSC derived “myeloid like” progenitor (iMye), expressing high levels of Runx1, PU.1, Irf8 and CD34, and lower or equivalent levels of MYB compared to blood monocytes.

In the different published protocols, since a few years, a number of them used in their initial steps the protocol from Wilgenburg et al. yielding progenitors expressing classical myeloid markers (Wilgenburg et al. 2013; Haenseler et al. 2017; Brownjohn et al. 2018; Garcia-Reitboeck et al. 2018). More recently, these progenitors were characterised more specifically regarding their Myb dependency or not during the differentiation. Interestingly, they showed that the progenitors they obtained were Myb-independent and Runx1-, PU.1-dependent (Buchrieser, James, and Moore 2017), a characteristic of YS-derived macrophages progenitors. Our results are in line with these reports, and thus confirm the possibility to generate myeloid progenitors in vitro which recapitulate some features of the in vivo cells.

An important point which emerged from the protocols subsequently published, and that we have also used in our protocol, is the induction of the differentiation using EBs. EBs recapitulate some of the morphogenetic interactions that are usually happening in vivo. In the protocol this first 3D culture step, ameliorates considerably the generation of myeloid progenitors by offering a more physiological embryonic-like environment as well as an hypoxic condition which was shown to be beneficial for early development. In fact, in vivo embryogenesis is occurring in relatively hypoxic conditions due to rapid cell proliferation and the increasing oxygen demand and consumption (Simon and Keith 2008; Dunwoodie 2009; Y. Gao and Raj 2010). Increasing interest has emerged as to mimic the oxygen tension in order to modulate cell proliferation. In vitro, low oxygen has

been demonstrated to play a role in the differentiation of stem cells into numerous of different cell fates (K.-M. Ng et al. 2010; Shin et al. 2011; Lesinski et al. 2012; Stacpoole et al. 2013; Binh et al. 2014; Podkalicka et al. 2020). Some protocols which are not using EBs in the initial step propose a 2D culture and modulation of oxygen concentration, which was shown to favour early cells differentiation (Pandya et al. 2017; Abud et al. 2017; Takata et al. 2017; Konttinen et al. 2019).

1.2.2 Maturation of iMye into iMG

Recent studies suggest that the cell origin may not be the only parameter which dictates macrophages/microglia identity and that the microenvironment could have major importance (Mass et al. 2016; Guilliams and Scott 2017).

In order to induce microglia maturation we thought to use direct contact with neuronal cells and adjunction of specific cytokines (IL-34 and TGF β 1) which were previously described as essential for microglia maturation.

Microglia essential factors for maturation

IL-34 is now well recognised to be a neuronal-linked cytokine necessary for microglia maturation (Y. Wang et al. 2012; Greter et al. 2012). Most of the published protocols used IL-34 for microglia maturation and so are we in the protocol we developed. Besides IL-34, more recently the attention has been driven onto TGF β 1 as an essential factor for microglial maturation in vivo and in vitro (Butovsky et al. 2014; Spittau, Dokalis, and Prinz 2020). TGF β 1 is secreted by astrocytes, and culture of iPSC derived microglia progenitors onto astrocytes was shown to be sufficient to induce microglia maturation (Pandya et al. 2017). Removal of TGF β 1 in iMG cultures conducted to deregulation of 1517 genes, with many of the genes affected being core microglia signature genes including P2RY12, TGF β R1 and CD33, showing the important role of TGF β 1 in microglia maturation (Abud et al. 2017). Some of the published protocols to differentiate iPSC into iMG do not use TGF β 1 for maturation (Muffat et al. 2016; Douvaras et al. 2017; Takata et al. 2017; Brownjohn et al. 2018; Garcia-Reitboeck et al. 2018; Claes et al. 2019; Konttinen et al. 2019; Guttikonda et al. 2021). It would be interesting to compare the transcriptomic profile of the iMG we have obtained using TGF β 1 with the iMG obtained in studies which did not use TGF β 1 during maturation.

Neuronal contact

In vivo, microglial cells develop in a highly specific environment that is the brain. They parallel the development of neuronal cells and highly contribute to their functional maturation. In vitro, direct contact of microglia with neuronal cells has proven to increase several markers linked to their core signature (Haenseler et al. 2017). SIGLEC11 and SIGLEC12 were reported to be increased in co-cultured microglia and are proteins having major roles in neuroprotection and suppression of pro-inflammatory signalling (Linnartz-Gerlach, Mathews, and Neumann 2014; Abud et al. 2017). Which we have also observed in our transcriptomic analysis. Additionally, in some protocols, the media supplementation with CX3CL1 and CD200 which are endothelial and neuronal proteins serving as off-signalling for microglia, lead to upregulation of genes linked to sensome, immune response, while maintaining the expression of core microglia signature genes (Abud et al. 2017). These findings further reinforce the hypothesis that neuronal environment is necessary for microglia maturation.

Comparison to human transcriptomic studies

In the protocol we have developed, we have matured iMye in contact with 7 days old iPSC derived MNs (iMNs) during 14 days. qRT-PCR analysis showed that microglia cells which were cultured over MNs had increased microglia core signature markers. Moreover, PCA of iMG culture over neurons (iMG+iMNs) or alone and blood monocytes derived macrophages (bmdM) showed that the first dimension of the PCA was able to discriminate the three different cell types with bmdM being at one extreme and iMG+MN the other, and iMG clustering in between.

The initial microglia homeostatic gene signature has been developed from mice studies. Although strong similarities between murine and human microglia have been demonstrated, several arguments suggest a divergence between species in their immune system (Bailey, Christoforidou, and Lewis 2013; Shay et al. 2013). Due to difficulties in accessing human material, studies which have sequenced the human microglia transcriptomes are scarce. Those who did it reported that human microglia expressed high levels of rodent known canonical microglial genes such as *CX3CR1*, *CD11b*, *P2RY12*, *Transmembrane Immune Signaling Adaptor (TYROBP)* (Gosselin et al. 2017; Galatro et al. 2017; Esaulova et al. 2020). Interestingly, a comparison between the human microglial transcriptome and three data sets of mice cortex microglia transcriptome, revealed an

important overlap between gene expressions, with some notable differences (Galatro et al. 2017; Gosselin et al. 2017). Amongst the genes that were more expressed in human compared to mouse microglia were genes implicated in the complement system, as well as brain structure in the data set from Gosselin et al. 2017. While in Galatro et al. study, was reported an increased expression in human microglia of genes mostly linked to host defense or the modulation of immune response such as: Lymphocyte function-associated antigen 3 (CD58), Endoplasmic reticulum aminopeptidase 2 (ERAP2), Granulysin (GNLY) and Protein S100-A12 (S100A12). More recently, single cell RNA sequencing allowed to demonstrate a diversity of microglial subtypes in human and rodent brains and suggested that a unique signature of microglial cell per say may possibly be too reducible (Mathys et al. 2017; Masuda et al. 2019; Velmeshev et al. 2019; Sankowski et al. 2019). Moreover, it is important to note that the majority of studies carried out on human microglial cells come from patients suffering from possible CNS pathologies and therefore that the transcriptomic signature does not necessarily reflect the basal state of these cells in a general way.

In our study, we aimed to characterise microglia based on the known homeostatic microglia gene signature extracted from different studies, and found that most of the genes were expressed by our cells. We also observed that some characteristic genes were expressed in the iMP but not at the same levels, in agreement with some other studies (Muffat et al. 2016; Haenseler et al. 2017; Chiot et al., 2020). P2RY12 and CX3CR1 were both highly expressed in our study and they are specific of iMG in our study. As they are known to be highly specific of microglial cells compared to macrophages, this reinforce the conclusion that the cells we generated in our protocols are microglia-like cells. However, we observed that the expression of Tmem119 was more expressed in bmdM and iMP compared to iMG+MN and iMG, conversely to a previous report (M. L. Bennett et al. 2016). Yet, in the study from Muffat et al. it is suggested that its levels of expression could differ between iPSC cell lines (Muffat et al. 2016). Moreover the exposure to serum could upregulate expression of Tmem119 as suggested the study from Douvaras et al. (Douvaras et al. 2017). Together these different results suggest that the expression of Tmem119 might not be a stable marker for microglia characterisation at least in vitro (Muffat et al. 2016; Douvaras et al. 2017). Single cell RNA sequencing would be interesting to be conducted on our iMG+iMNs and iMG cultures, in order to reveal if different iMG subtypes coexist in vitro.

1.2.3 Maturation of iMye into iPSC derived macrophages (iMP)

In the literature, in vitro macrophages are often matured with M-CSF or GM-CSF. GM-CSF promotes a more pro-inflammatory phenotype while M-CSF induce a more anti-inflammatory phenotype (Sica and Mantovani 2012; Xue et al. 2014). In our study, we used M-CSF to mature our iMye into iMP and the PCA showed that the iMP clustered closely to bmdM. Moreover, we have characterised these cells at different levels. iMP were expressing macrophages characteristic genes including CCR2, Clec12a, Lyzl2, Tgfb1, Clec10a, Emilin2, Gda, Hp, Sell (Haage et al. 2019; Chiot et al. 2020) which were reported to be specific in MP in mice. With regards to human cells, most of the studies which characterised the expression of macrophages used blood monocytes that were matured in culture and thus might not recapitulate the in vivo macrophages signatures (Beyer et al. 2012). To my knowledge, there is no studies, characterising the transcriptome of human peripheral tissue-resident macrophages. From our analysis, we can say that iMP matured alone using M-CSF cluster with bmdM at the transcriptomic level, and that the iMP we have generated display some MP specific signatures identified in mice studies.

As stipulated with microglial cells, we sought that the microenvironment may also change the maturation state of our macrophages. In order to prevent dedifferentiation of macrophages into microglia, we have first cultured our macrophages during 7 days and detached our cells and cultured them over neuronal cells. We observed that in fact the transcriptomic profile was also changing, and pathway analysis demonstrated changed in leukocyte trans endothelial migration, regulation of actin cytoskeleton, ribosomes and cell cycle suggesting that the environment may also, contribute to macrophages maturation. Finally, our transcriptomic study showed that iMG and iMP cultured over iMNs both clustered separately, and harbour specific gene signatures which were in line with published transcriptomic studies, supporting that the two cell types we have generated are different. These findings have permitted us to validate our protocol for usage to study iMP and iMG respective roles towards ALS MNs degeneration.

1.3 Co-cultures between iPSC derived innate immune cells and motor neuronal cells

In order to study the role of ALS iMP and iMG on MNs survival we have established co-cultured between the different cell types. The main challenge in this development was the synchronisation of the different protocols in order to be able to compare the same time points between different

experiments. We sought to study the role from an early time point when MNs are still healthy to a later time point at which ALS MNs start demonstrating disease phenotypes (Lefebvre-Omar In preparation).

iMG cultures were established from the moment the progenitors were harvested and directly matured onto the MNs in order to mimic the neuronal development. As immune cells are easily polarisable due to their numerous membrane receptors, we aimed to realise a negative magnetic sorting to collect our cells using the p75 Neural Crest stem cell marker. We have realised qRT-PCR to measure Islet1 mRNA and we verified that only the positive fraction contained Islet1, showing that our iMG analysed in RNA sequencing were pure.

iMP co-cultures were more challenging, as we had to adapt our protocol. Firstly, macrophages were matured in mono-cultures and subsequently when they were differentiated 7 days we have detached them from the plate and plated them over MN cells. As expected, we have observed that most of the iMP maintained their elongated morphology. Similarly, negative sorting was realised to collect the cells for RNA sequencing to avoid activation of cells by the beads.

With this model, I was able to ask important questions about the roles of macrophages and microglia in ALS. One limitation remains regarding the mimicking of the physiological conditions, especially for macrophages. Indeed macrophages are not in contact with MN soma in vivo. The contact of macrophages with soma MNs in our culture model may have induced a specific response despite the differences between iMP and iMG we found in our analysis.

In order to remedy to this limitation we sought to implement microfluidic devices with separate compartments (see below). This way of implementing co-cultures is particularly interesting to study different cells interactions in a human context.

1.4 Microfluidic chambers to physically separate soma from axons and better study the implication of the different immune cells on their cell targets

The implementation of microfluidic chips was a long-term work strewn with obstacles. I spent 3 years trying to design the best chip model for my experiments. Unfortunately, I was unable to use the chips once set up due to lack of time. Nonetheless, I will use them for my experiments in the

next months to compare co-cultured results with the ones obtained with compartmentalised cultures, and to study synergic effects of both macrophages and microglial cells when cultured in the same chip with MNs soma and axons in different compartments.

In collaboration with Dr. Peyrin, Dr. Courte and Pan Teng (PhD student in Dr Peyrin's lab), we first started with "classical chips", which permit the strict isolation of soma and axonal compartments. Unfortunately, important axonal degeneration was observed early on in the chips, which we identified being linked to the fact that grooves were too narrow for iPSC derived MNs.

Furthermore, when we added iMye in the chips for maturation into iMG and iMP, we rapidly observed migrating cells going from the axonal chamber to the soma chamber and less the other way around. To prevent this backward migration we adapted a chip which has been developed by Dr. Courte in the laboratory (Courte et al. 2018). The asymmetrical design, which they used as a "return to sender" strategy for neuronal cultures, showed high efficacy to prevent macrophages from migrating backward towards MNs soma. This migration phenomenon was less present with microglia cells, which mostly stayed in the soma compartment. This was interesting as it suggested us that soma compartment may secrete factors that attracted macrophages.

Another trouble in our work was the observation that migrating cells were often presenting more rounded morphologies. We know that microglia and macrophages phenotypes and adhesion properties are highly influenced by external cues (McWhorter, Davis, and Liu 2015). In the microfluidic chambers, we observed that due to the important influx generated during media change most of the macrophages and microglia rolled out the axonal compartment and seeded over in the media loading space. Thus conducting to progressive depletion of macrophages and microglia in the different compartments. Thanks to Dr. Peyrin and Pan Teng, a modification of the chips was designed, finding a way to control the influx flow while allowing sufficient oxygen input for cells.

These prototypes have been now tested and validated several times and further experiments will be conducted in these devices in order to confirm our observations. Altogether, these new models are particularly interesting to allow more specific culture of microglia as well as macrophages with

MN subcellular compartments. Adding this precision allow us to better mimic the in vivo interactions and microenvironment unique to each cell type. Supported by the study from Chiot et al, which showed that the replacement of ALS peripheral macrophages with more trophic macrophages extend G93A SOD1 mice survival (Chiot et al. 2020); we could imagine using the microfluidic devices in order to see if strict modulation of various ALS forms macrophages in the axonal compartment can benefit MNs survival as well as the effects on the “well-being” of the soma compartment.

In the studies conducted to date using microfluidics in ALS, a number of them attempted to model neuromuscular junctions using iPSC derived myocytes and to study the role of neuromuscular junctions defects in ALS disease (Chennampally et al. 2021; de Jongh et al. 2021); but none has yet studied the role of innate immune cells in ALS using microfluidics. The perturbation of neuromuscular junctions has been shown to be an early event in ALS disease in mice but also in ALS patients, preceding symptoms onset (Carvalho and Swash 2013; Cappello and Francolini 2017). Additionally, some studies evidenced an important role of macrophages in ALS neuromuscular junctions integrities (Al-Sarraj et al. 2014; Pollari et al. 2014; Van Dyke et al. 2016), without determining their exact trophic or toxic role in the neuromuscular junction deterioration. Thus as interesting perspectives of my work, we could imagine combining the generation of neuromuscular junctions with the addition of innate immune cells in microfluidics, which would allow us to better understand the role of these cells in neuromuscular integrity and support. ALS neuromuscular junction loss being an early event in the disease identifying targets which could be modulated at this level would permit to delay or prevent neurodegeneration and subsequent paralysis of ALS patients.

2- The temporality of inflammatory events in ALS

2.1 Secretory profile in ALS patients: where are we?

In ALS a large panel of pro- and anti-inflammatory interleukins and chemokines have been identified as deregulated in patients biofluids compared to controls (See introduction). However results are not always consistent amongst the different studies mainly due to the heterogeneity of the studied population (X. Chen et al. 2018, Hu et al. 2017). The latter heterogeneity is seen at several levels:

- (i) In most studies, included patients are diagnosed with El Escorial criteria and categorised as possible, probable, definite disease. As this way of classification can be interesting to study correlation of patients between the diagnosis and the amount of circulating factors; it holds limitations in studies which are pooling the different diagnoses into one group and that study the circulating inflammatory factors as a whole in this unique group.
- (ii) Moreover, in most studies, patients are seen at one single time point and at a variable state of their disease (ALSFRS-score with large standard deviation). These patients may thus be in different states of inflammation (if referring to studies in rodent models: the early anti-inflammatory phase vs the late pro-inflammatory phase) (Hooten et al. 2015), making the results hard to interpret. Also, the state of disease often measured with the ALSFRS scale, does not necessarily reflect the state of the pathology at the cellular level. To date the most promising biomarkers for ALS are heavy and light chains neurofilaments, which are measured in CSF and blood samples (Xu 2016) and could represent a more robust way to assess the state of the disease.
- (iii) The site of onset of the pathology (spinal vs bulbar) may also be a confounding factor. Indeed progression of the disease is very different between the two forms of the pathology, and so are probably the inflammatory events over time.
- (iv) In most studies sporadic patients are studied, which might underline very different pathologies. Moreover, some studies are also comparing familial cases with sporadic cases. Knowing the importance of some mutation in the immune system, retrospective studies may be interesting to isolate subtypes of patients, which are responding more similarly.
- (v) Lastly, biofluids which are assessed are also different depending on the studies. Some are measuring CSF, while others blood. Circulating cytokines and chemokines in these biofluids may be different depending on the state of the pathology, disease onset and other factors thus are difficult to be compared.

To date very few studies conducted longitudinal studies. From those who attempted to follow up their patients no significant differences of cytokines secretion was observed between the two time points (Saleh et al. 2009; Ehrhart et al. 2015b; Prado et al. 2018). However, in most cases recruited

patients were already advanced in pathology. Thus if inflammation is an early event, such studies may have missed the critical points at which real differences may be seen.

Some studies have started to categorise their patients according to disease progression rates (fast vs slow) or different genetic forms, and interestingly using this sub classification, some factors were found to be correlated with disease progression and prognostic (F. Huang et al. 2020; Olesen et al. 2020). Thus supporting the idea that specific inflammatory events may underlie ALS subtypes and strengthening the need to better identify the temporality of these events in order to offer phenotype specific therapeutic options.

2.2 Secretory profile over time in iPSC derived MG and MP cultured with MNs

In our study, we aimed to investigate the secretory profiles of mixed and matched ALS iMG and iMP cultures over MNs. As underlined by the over mentioned discussion the temporality of the events being important, we aimed to assess the changes over a period of 11 days in culture corresponding to a delay during which MN degenerate when they are alone. Additionally, the selection of the time points were supported from a previous study conducted in the laboratory by Dr Bohl's group. In fact, at the later time point, ALS MNs were shown to already present some characteristic pathological defects including proteins aggregations, defects in excitability and slight neuronal suffering (Lefebvre-Omar et al., *).

In co-culture supernatants, we have measured 26 different inflammatory factors at the two different time points. Interestingly, from these studies we have isolated three different types of inflammatory responses which will be discussed herein.

2.2.1 Undetected inflammatory factors in co-cultures

Absence or very low secretion of certain cytokines by neither the immune cells nor the MNs suggest that the environment did not stimulate these responses. While some factors were absent in both cell type cultures including: some IL-1 family cytokines (IL- β , IL-18, IL-33) (Dinarello 2018), IL-12p70, IL-17A, IL-23, IFN γ , IFN α 2, TNF α , sTREM1, CXCL12, sST2, sRAGE and CX3CL1; some other factors were expressed only in one or the other cell type and thus were more cell-type specific arguing in favour of the fact that the two immune cell types generated in our cultures were different and had distinct basal secretory profiles. For instance in iMP cultures there was no secretion of TGF β 1 compared to significant levels in iMG, while in the latter no sCD40L and sCD25 were measured compared to iMP.

IL-1 family cytokines secretions has been reported in the literature in both patients and animal studies, although reports from ALS patients' biofluids do not present consensus (Table 2, 3, 4, 5 in the introduction). Over the studies measuring IL-1 β in the CSF (n=4 studies) and blood (n=5 studies) of patients, only two independent studies reported increased IL-1 β in ALS patients CSF and blood (Tateishi et al. 2010; Lu et al. 2016). Regarding IL-18 in the CSF and blood, two studies reported increased concentrations in ALS patients compared to controls (Italiani et al. 2014; F. Huang et al. 2020). In the G93A SOD1 mouse model, it has been previously shown that mutant SOD1 activated caspase-1 and IL-1 β in microglia. And that modulation of this response by deficiency in caspase-1 or antagonist of IL-1 receptors extended the survival of ALS mice and attenuated inflammatory pathology (Meissner, Molawi, and Zychlinsky 2010). A clinical trial in ALS patients have used recombinant human IL-1 receptor antagonist to see if the modulation of IL-1 response could be beneficial. Disappointedly, this study did not prove any long term efficacy in patients (A. Maier et al. 2015).

In our study the fact that the IL-1 response was not detected (or below the detection level) suggested that the contact of ALS iMG or iMP with ALS iMNs was not sufficient to induce IL-1 response at this stage of culture. However, when we stimulated mono-cultured cells with LPS (10ng/mL) we observed IL-1 β secretion (not shown), suggesting that the IL-1 capability of secretion was not impaired in our cells. Several other raisons explain why we did not detect any IL-1 β :

- (i) In vivo, several inflammatory factors are circulating and contribute to the priming or activation of the innate immune cells. For sure, in our co-culture conditions effects coming from other neighbouring cells could not be measured, as we focused on binary interactions between MNs and macrophages or microglial cells. In future experiments we could use external activators, such as addition of LPS or protein aggregates, to mimic external insults and measure secretory profiles in these conditions, as well as quantify the neuronal network integrity in response to the stimulation.
- (ii) The time points of our analysis are maybe too early to detect any IL-1 response. In fact Olesen et al. reported that IL-1 β secretion was more specific to patients

in late disease stage (Olesen et al. 2020). It would be interesting to measure cytokine secretions at a later time points in order to see if other inflammatory factors are secreted including IL-1 β and other IL-1 family factors.

- (iii) In most ALS patients' studies, sporadic cases are included. IL-1 family cytokines may be specific to inflammatory responses from some ALS patients subtypes. In the laboratory we dispose of iPSC lines from sporadic ALS patients. Our studies will be replicated using these iPSC lines in order to compare our current observations.

In regards with the past studies conducted in ALS patients' biofluids, we witness that similarly to our observations numerous factors were not detected in patients' biofluids. The fact that some studies but not others observed secretions of certain factors demonstrates once again the importance of the temporality of inflammatory events and reinforces the need for a better classification of patients and longitudinal studies. For instance, the studies by Huang et al. and Olesen et al. showed that C9orf72 ^{(G4C2)ⁿ} patients have distinct secretory profiles compared to other ALS forms. Differences in secretory profiles were also seen between fast and slow progressors (Olesen et al. 2020; F. Huang et al. 2020).

2.2.2 Canonical secretory profiles of iMP and iMG co-cultured with degeneration of iMNs over time

During the inflammatory response, first line effectors are innate immune cells. They respond rapidly to the damaged tissue by releasing pro- and anti-inflammatory mediators, which direct the inflammatory response towards inflammation or resolution (Askenase and Sansing 2016). In pathological condition, some inflammatory markers can also be secreted; however, it is difficult to delineate the beneficial from detrimental responses.

Firstly, we sought to characterise the "classical" responses, of macrophages and microglial cells when they were in contact with MNs. In order, to be able in a second step to better identify the pathological responses in mutant cultures (see below).

We witnessed that the secretory profiles of iMP and iMG in contact with iMNs were evolving over time. This suggests that the innate immune cells were responding to the motor neuronal environment. Interestingly, we observed an early and a late response, which were underlined by

the presence of different factors. Although iMP and iMG showed some similarities in the factors secreted over time in culture, it appeared that some responses were more cell type specific (Figure 21, Figure 22).

The absence of neurotrophic factors in the media we used in these co-cultures conditions induced more rapid neuronal suffering compared to MNs cultured alone. However, in all co-cultures conditions no cellular debris were observed demonstrating an active clearance activity of macrophages and microglia which certainly contributed to their secretory profile over time.

The early response in both iMP and iMG co-cultures with MNs was shown to be governed mainly by IL-10, IL-8, IL-6 and MCP-1 (Figure 21, 22).

- IL-8 is a chemoattractant mainly for neutrophils which are often the first cells recruited at the site of injury (Russo et al. 2014). In fact, in iMP and iMG cultures an important increase of IL-8 secretion was observed which decreased with time (Figure 21 and 22). In conjunction with IL-8 secretion, MCP-1, a chemoattractant for leukocytes was shown to be highly upregulated early on in iMP and iMG. We observed that, in iMG cultures the level of MCP-1 was maintained over time suggesting that iMG possibly reacted with a more pro-inflammatory profile along MNs degeneration, and induced more attraction towards peripheral cells to resolve inflammation.

This specific iMG response suggests that CNS and PNS inflammatory responses overtime may be different. In fact, in the CNS the most widely present immune cells are microglia representing 80%, the rest being myeloid cells, monocytes macrophages, dendritic cells, T cells, B cells and natural killer cells (Korin et al. 2017). We could here hypothesize that microglial cells upon injury aim to recruit number of other immune cells which would help for the resolution of the injury, yet due to limited number of other immune cells in the CNS the response is sustained much longer than in macrophages which are located in the PNS. Another hypothesis could also be that the injury begins in the soma level.

Interestingly, almost all studies in patients, that assayed MCP-1 in the CSF, found a level higher than the controls (R. M. Mitchell et al. 2009; Kuhle et al. 2009; Tateishi et al. 2010; Furukawa et al. 2015; J. Guo et al. 2017; Gonzalez-Garza et al. 2018). In addition, some

studies in ALS patients post mortem tissues have shown infiltration of dendritic cells and monocytes in the spinal cord, in conjunction with increased MCP-1 level and activated microglial cells (Wilms et al. 2003; Henkel et al. 2004). More recently, MCP-1 response was also shown to mediate NK cells recruitment in the spinal cord of G93A SOD1 mice (Garofalo et al. 2020). In our study, we did not report an increase of MCP-1 in mutants cultures compared to controls. This could be linked to the fact that we analysed our cultures too early, and that longer cultures may be necessary in order to observe differences between controls and mutants. This could also mean that we need to add in our cultures the cell types targeted by the MCP-1 to assess whether they would be attracted and if MCP-1 secretion would be modulated.

- IL-10 is the most widely accepted anti-inflammatory cytokine (Sabat et al. 2010). We observed that in culture IL-10 secretion was increased early on for both iMG and iMP co-cultures. However, the level decreased over time in iMG culture but remained stable in iMP cultures. This suggests that in the initial phase innate immune cells could elicit an anti-inflammatory response when they enter in contact with MNs. Yet the fact that IL-10 secretion is maintained in iMP suggests that over time iMP sustain this anti-inflammatory phenotype. IL-10 can also exert indirect effects over time, with for instance the induction of the release of anti-inflammatory mediators such as soluble TNF- α receptors which include sTNFR-I and II (Jenkins, Malyak, and Arend 1994; Joyce et al. 1994; Hart et al. 1996). We hypothesise that this could be the case in our cultures as we observed increased of both of these factors over time in iMG and iMP cultures. IL-10 was also shown to be able to increase the phagocytosis of macrophages/microglia (Buchwald et al. 1999; Sabat et al. 2010). Given the multifaceted role of IL-10 in calming inflammation, some studies are now considering the modulation of IL-10 in several chronic diseases including Bowl disease, rheumatoid arthritis, and systemic lupus erythematosus (X. Wang et al. 2019). Maintenance of the IL-10 concentration may be interesting to be tested on long-term ALS iMP+iMN or iMP+iMB co-cultures conditions to assess the potential beneficial anti-inflammatory reaction over time towards ALS MNs survival.

- IL-6 is a pleiotropic cytokine, which has both anti- and pro-inflammatory functions. Its main anti-inflammatory functions are mediated via the membrane bound IL-6 receptor and act on STAT3 pathway leading to cell proliferation and inhibition of apoptosis. On the other hand, the pro-inflammatory action of IL-6 is linked to the soluble IL-6 receptor (sIL-6R) via trans signalling pathway. In vivo this contributes to the activation of the immune system by recruitment of mononuclear cells and modulation of the adaptive immune system (Scheller et al. 2011). In our cultures, we could speculate that IL-6 has an early anti-inflammatory role acting via the membrane bound IL-6R, which would over time possibly become more pro-inflammatory (this will be discussed in the next part). This hypothesis of an early anti-inflammatory role of IL-6 could be studied using specific membrane bound IL-6R antagonist. The fact that IL-6 can have both anti and pro-inflammatory effects is confounding in ALS disease as it is hard to identify if the response is beneficial or detrimental. Some studies reported increased levels of IL-6 in the CSF and plasma of ALS patients (Moreau et al. 2005; R. M. Mitchell et al. 2009; Ehrhart et al. 2015a; Furukawa et al. 2015; Lu et al. 2016; Blasco et al. 2017b; Prado et al. 2018). It thus remains to be determined if these observations are only reflecting an ongoing beneficial inflammatory response or if it drives ALS disease progression.

While the early phase response seemed to be quite similar between iMG and iMP, the late phase response appeared to have cell type specificities. The factors that are more specific to the late phase response and are shared between the two cell types are an increased level of sCD130, sTNFR-I and II. With regards to cell type specific factors we noticed increased sCD25 in iMP, and increased TGF β 1 only in iMG over time.

In addition to some new factors that were secreted between the two time points, we observed that some factors were also maintained over time. This included MCP-1 in iMG (already discussed in the previous section), and there was also IL-6 that seemed to be more iMP specific.

Macrophages specific response

- IL-6 trans signalling via the soluble IL-6 receptor was shown to play an important role in chronic inflammation. Interestingly, soluble CD130 (sCD130) was shown to act as a natural inhibitor of soluble IL-6 receptor trans signalling (Jostock et al. 2001). Nevertheless, at high concentration sCD130 can also interfere with classical IL-6 signalling via the membrane

bound IL-6R. Interestingly, while the level sCD130 increased in both iMG and iMP, the level of IL-6 was shown to decrease over time. This suggests that IL-6 pro-inflammatory function may be dampened by the increased release of sCD130. This reaction may be beneficial in order to prevent inflammation to be chronic and deleterious for MNs survival.

- sCD25 is a soluble IL-2 receptor α . Upon activation of immune cells, surface CD25 is cleaved by proteases including matrix metalloproteinase-2 and 9 (MMP-2, MMP-9) (Kniep, Strelow, and Lohmann-Matthes 1992; Rubin et al. 1990; Brusko et al. 2009). In vitro studies showed that sCD25 at low concentrations potentiate IL-2 signalling (Z.-Z. Yang et al. 2011), while high concentrations block IL-2 signalling (Brusko et al. 2009; L. M. Maier et al. 2009; Russell et al. 2012). IL-2 also promotes Tregs differentiation, cells which were shown to play a major role in ALS disease. Interestingly, low dose IL-2 infusion in ALS patients is being investigated as a phase IIa clinical trial (Camu et al. 2020). Its secretion during the second phase of the inflammatory response demonstrates that it may have a role to support MNs survival. Yet due to the complexity and its dual roles, it is difficult to draw clear conclusions. Modulation of its signalling would appear interesting in our cultures in order to observe changes on MNs survival, and better understand its contribution towards MNs support in control conditions. Interestingly as sCD25 is mainly having a role on T cells, it is interesting to observe that it was mainly increased in macrophages which are more in contact with adaptive immune system cells than microglia. This suggests a more peripheral specific immune response, which will be interesting to validate in microfluidic devices in order to see if the response is also present when cells are only in contact with MNs axons.

Microglia specific response

- ❖ Interestingly, TGF β 1 as discussed previously promotes a trophic support for microglial cells, and contribute to a more anti-inflammatory response. The fact that in our media we have used TGF β 1 for microglial maturation renders difficult any clear conclusion. As it is hard to separate the effects being linked to the presence of TGF β 1 in the media from that of a physiological response of the immune cells to the micro-environment. Nonetheless, when we have measured a basal level of TGF β 1, we still observed its increase in the co-cultures conditions, suggesting either that microglial cells were in fact mediating an anti-

inflammatory response independently of the presence of TGF β 1, or that the presence of TGF β 1 in the media triggered an autocrine feedback loop conducting to sustained production of TGF β 1. However, an interesting observation is that in different ALS iMG the secretion levels were variable and seemed to be mutation specific, thus demonstrating that the sole presence of TGF β 1 in the media was not sufficient to maintain this feedback loop. To go a step forward, we observed that the fast progressing SOD1^{H44R} iMG had very low levels of TGF β 1 while the slow progressing SOD1^{N139D} iMG presented increased TGF β 1 level already in the early phase and an increasing trend in the late phase, suggesting that the secretion of TGF β 1 could be a protective factor in ALS disease progression. Nonetheless, further analysis have to be conducted to confirm or not these findings and hypothesis. In support with these hypothesis, several studies supported the importance of altered TGF β 1 with aging and in ND such as AD, and its subsequent reduced protection against pro-inflammatory stimuli (J.-H. Chen et al. 2015; X.-X. Fang et al. 2018; Hodgson et al. 2019). We cannot preclude that our observations are linked to the presence of TGF β 1 in the media. In future experiments we will substitute TGF β 1 by Inducer of Definitive Endoderm 1 (IDE1) a factor mediating the phosphorylation of TGF β downstream signalling to mimic the TGF β 1 and promote microglial maturation (Borowiak et al. 2009; McQuade et al. 2018).

Present in both macrophages and microglia

- sTNFR-I and sTNFR-II soluble TNF α receptors compete with the transmembrane TNFR forms and thereby inhibit TNF α . TNFR-I receptor is ubiquitously expressed and has mainly been associated with pro-inflammatory signalling due to its role in apoptosis. Whereas TNFR-II is mainly expressed in T cells and stimulates NF- κ B signalling and displays more anti-inflammatory functions (Faustman and Davis 2010; Paccalet et al. 2021). We have been surprised to observe the increase secretion of these two factors without any major increased concentration of TNF α in our cultures. Either the receptors were scavenging TNF α and we thus could not detect any secretion, or these receptors had other undertermined responses. In the study of Olesen et al. TNF- α secretion was associated with late disease stage ALS patients (Olesen et al. 2020). Thus, later time points cultures will be conducted to see if over time we observe an increased secretion of TNF α . Nonetheless, as sTNFR-I and sTNFR-II were proven to increase with time in our cultures, it

reinforces the idea that over time the anti-inflammatory signalling tend to support MNs survival in control conditions.

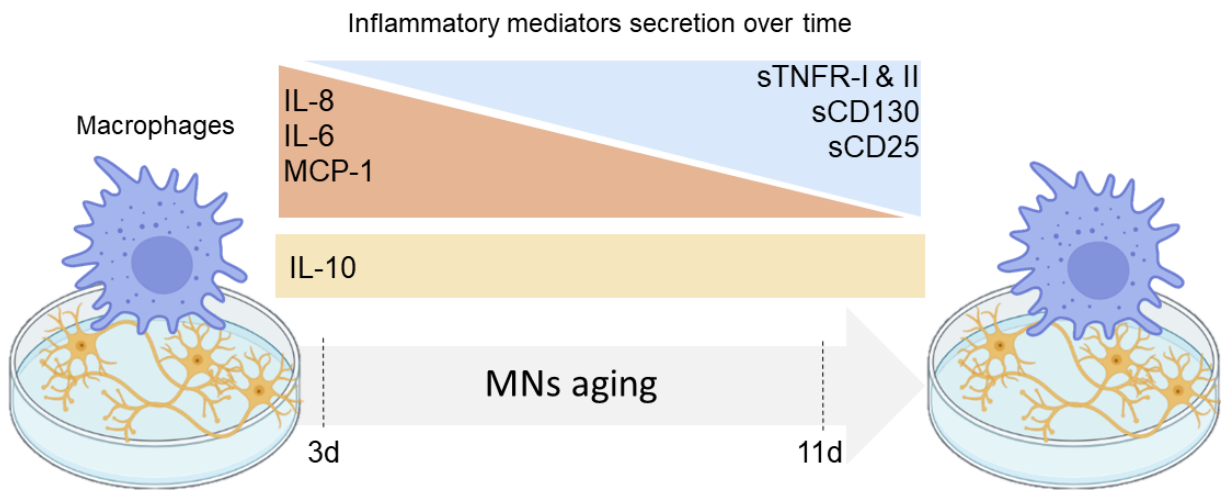


Figure 21. Macrophages ctrl and mutant secretion profile over time

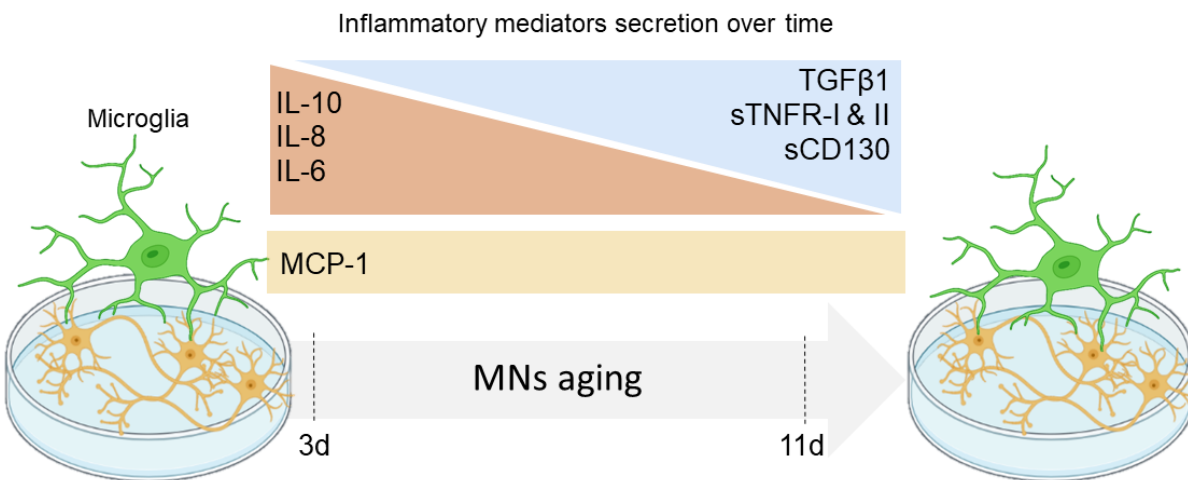


Figure 22. Microglia control and mutant secretory profile over time

The identification of this temporal response to neuronal suffering is particularly interesting, because it makes it possible to hypothesize the bases of generic responses found in patients which could correspond to a beneficial phase of the pathology in which the immune system is trying to resolve the injury. However, if the response is prolonged it could lead to over activation of the immune system which could contribute in ALS pathogenesis. As a parallel with the simplified categorisation of the two extreme polarisation of macrophages/microglial cells, we observed that our co-cultures models seem firstly presenting a balance between pro-inflammatory response

mediated by the increased secreted chemoattractants, and an anti-inflammatory response corresponding to the presence of IL-10. In conjunction the role of IL-6 is for the moment still difficult to delineate, but could be supposed to be more anti-inflammatory in this early phase. Interestingly, this initial response phase is shared by both cell types suggesting that it may represent a generic response upon activation of immune cells. In the second phase of the response, we observed that the secretory profile differed between iMG and iMP, although both secreted sCD130, sTNFR-I and II. iMG during late phase response presented increased TGF β 1 which has a role in anti-inflammatory cascade, yet the MCP-1 response was still maintained at an important level suggesting again a balance between pro- and anti-inflammatory responses. Whereas in macrophages the cell type specific responses were underlined by secretion of sCD25 which function is mainly concentration dependent and which was shown to be increased, in parallel a sustained level of IL-6 concentration was observed which is possibly controlled by the increased level of sCD130. Similarly, to iMG, a balance between a mostly anti-inflammatory response based on sCD25 low dose which could promote Tregs expansions, and a pro- or anti-inflammatory response by IL-6 seemed to govern the iMP late phase response to MN aging in culture.

The cellular debris generated by axons suffering over time could mediate a DAMPs response cascade, although it did not culminate with the activation of inflammasome as no IL-1 β nor IL-18 were detected in the supernatants. We could speculate that with time the accumulation of neuronal suffering may trigger a more pronounced inflammatory response.

2.2.3 ALS mutant specific secretory profile over time

In ALS disease, most of the understanding regarding inflammatory sequential responses has been described in G93A SOD1 mice, which may not be applicable to the different subtypes of ALS. Additionally, the understanding of the temporality of the inflammatory events in the pathology is important in order to better understand the time window, which offer the most promising results.

We have realised mixed and matched cultured of ALS and control iMP or iMG with iMNs, and compared the temporality of inflammatory mediators secretion over time. We observed different profiles of dysregulation between mutants but also between iMP and iMG.

Comparison between mutant iMP and iMG secretory profiles

Interestingly, we have observed that among ALS iMP co-cultures the most important deregulations in the secretory profiles were those with iMP mutated in C9orf72^{(G4C2)ⁿ}, SOD1^{H44R} and TARDBP^{G348C}; while among ALS iMG co-cultures the most deregulated was one was with SOD1^{N139D} iMG. These observations suggest that inflammatory reactions could be specific to one cell type rather than other, to one mutation rather than the other, and could be temporally regulated. These results are summarised in Table 11.

One satisfactory hypothesis to explain the differences observed between SOD1^{H44R} iMP and SOD1^{N139D} iMG is linked to the fact that patient carrying mutations in SOD1^{H44R} and SOD1^{N139D} have rapid and slow diseases respectively. Thus, the response we observed in the SOD1^{N139D} iMG may thus be a protective response the mutated iMG have developed in order to protect MNs. Several lines of evidences in our culture support this hypothesis. In fact most of the cytokines that are highly increased in these cells are anti-inflammatory mediators such as IL-10, TGFβ1, sTNFR-I and II. In conjunction we observed an increased sCD130 secretion level which is known to act as a natural inhibitor of IL-6 signalling, and thus could also contribute to a more anti-inflammatory response. Additionally, IL-8 is also increased, although IL-8 represent a more pro-inflammatory cytokine its secretion may suggest the importance of recruitment of other cell types at the site of inflammation in order to resolve the injury. For the moment, these arguments remain only hypotheses, which would be interesting to me to be deepened. Indeed, it is often a question of identifying the toxic mediator in order to prevent it from acting, but that said, the identification of beneficial factors as trophic support for neuronal survival could also represent a major interest for ALS patients.

Interestingly when comparing TARDBP^{G348C} iMG and iMP responses, we observed that they were opposite. TARDBP^{G348C} iMP presented decreased IL-8, MCP-1, sTNFR-I and II secretions compared to controls, suggesting less anti-inflammatory responses, while in TARDBP^{G348C} iMG an increased secretion of anti-inflammatory factors was observed compared to controls. This cell type specific secretion profile was also witnessed in C9orf72^{(G4C2)ⁿ} and SOD1^{H44R} iMG and iMP, suggesting that the two innate immune cell types can contribute differentially to MN support or degeneration.

Differences between ALS mutants

With regards to the different ALS mutants, we observed two main types of profiles. One with increased levels of some of the key factors detailed above in the “classical” inflammatory response (IL-10, IL-8, IL-6, MCP-1), and one with decreased levels of these same factors.

The four C9orf72^{(G4C2)_n}, as well as SOD1^{N139D} and SOD1^{H44R} patient’s cells presented either increased levels or similar levels of IL-10, IL-6, IL-8, MCP-1 compared to controls in both iMP and iMG, although concentrations and deregulations were cell type specific. This suggest that for these mutant the “classical” inflammatory response may be heighten in comparison to control cells. Whether this response increase is linked to mutant MNs suffering overtime, this seem to be factor specific. In fact in our mixed co-cultures over control MNs, we observed that for some of the cytokines (iMG: IL-10, IL-8. iMP: IL-10, MCP-1), the secretion levels appeared to be MN independent and may constitute an inner priming of the cells possibly linked to the mutation. This will be confirmed with the isogenic controls.

Regarding TARDBP^{G348C} mutants (mostly iMP), we observed that this response was in contrary to other mutants, decreased compared to controls iMP. This holds true for IL-10, IL-6, IL-8 and MCP-1 in iMP. If this decrease is deleterious for MNs survival remains to be determined more precisely.

Regarding factors that were dampen in mutants compared to controls in iMP we observed decreased release of most of the soluble receptors, which are thought to have mostly anti-inflammatory roles herein. This includes sCD25 secretion in C9orf72^{(G4C2)_n}, SOD1^{N139D}, and TARDBP^{G348C} patient’s cells, but not in SOD1^{H44R} patient’s cells; sCD130 secretion in C9orf72^{(G4C2)_n} and TARDBP^{G348C} iMP; sTNFR-I in C9orf72^{(G4C2)_n}, SOD1^{N139D} and TARDBP^{G348C} patient’s cells and a decrease in all ALS iMP for sTNR-II secretion compared to controls. Knowing that soluble receptors have concentration specific effects, both increased and decreased levels may lead to harmful deregulation and possibly contribute to MNs degeneration. However, this still has to be confirmed by directly modulating these factors in order to observe the consequences in MNs survival.

Taken as a whole, although major variations are observed between cell types as well as ALS mutations, we can clearly stipulate that there is overall deregulation of the inflammatory response

that seems to be partly governed by intrinsic defects possibly linked to the ALS mutations, but also an activation coming from the mutant MNs. Being able to isolate these different factors represents a particular interest in order to better orientate a future targeting. Moreover, similarly to control cultures we discerned an evolution of the inflammatory response over time in the mutant cells, with some factors rather present at the beginning of the culture and others at the late time point.

Table 11 Summary of the secretory profiles after 3 and 11 days of cultures of ALS mutants iMP and iMG with matched iMNs compared to control matched respective co-cultures.

| | iMP+ iMN | | iMG+ iMN | |
|---------------|---|--|---|---|
| | 3 days | 11 days | 3 days | 11 days |
| IL-10 | mC9orf72 \uparrow **** SOD1 ^{H44R} \uparrow **** | SOD1 ^{H44R} \uparrow **** | SOD1 ^{N139D} \uparrow ** TARDBP ^{G348C} \uparrow * | SOD1 ^{N139D} \uparrow * |
| IL-8 | mC9orf72 \uparrow ** TARDBP ^{G348C} \downarrow **** | | SOD1 ^{N139D} \uparrow ** | |
| MCP-1 | TARDBP ^{G348C} \downarrow | | = | = |
| IL-6 | = | = | SOD1 ^{N139D} \uparrow **** | |
| sCD130 | = | = | | mC9orf72 \uparrow * SOD1 ^{N139D} \uparrow *** |
| sTNFR-I | | TARDBP ^{G348C} \downarrow ** | | SOD1 ^{N139D} \uparrow *** |
| sTNFR-II | | mC9orf72 \downarrow **** TARDBP ^{G348C} \downarrow ** | | SOD1 ^{N139D} \uparrow ** mC9orf72 \downarrow * |
| TGF β 1 | = | = | SOD1 ^{N139D} \uparrow *** | SOD1 ^{H44R} \uparrow ** TARDBP ^{G348C} \uparrow ** |
| PAI-1 | TARDBP ^{G348C} \uparrow **** | mC9orf72 \uparrow **** SOD1 ^{H44R} \uparrow ** TARDBP ^{G348C} \uparrow **** | | SOD1 ^{N139D} \uparrow *** TARDBP ^{G348C} \uparrow ** |
| PTX-3 | | mC9orf72 \uparrow **** SOD1 ^{H44R} \uparrow **** TARDBP ^{G348C} \uparrow *** | = | = |
| sCD25 | | mC9orf72 \downarrow * SOD1 ^{H44R} \uparrow *** TARDBP ^{G348C} \downarrow * | Not secreted | Not secreted |

Mutant specific factors

We have identified two factors that were found to be secreted only in mutant cultures and not in control ones. These factors are Plasminogen activator inhibitor-1 (PAI-1) and Pentraxin-3 (PTX3). While PAI-1 seemed to be common to both cell types, PTX3 appeared to be more iMP specific.

PAI-1 is the main physiological inhibitor of plasminogen activators (PA) (Figure 23). In the CNS, PAI-1 has an anti-apoptotic role in neurons by acting as inhibitors of tPA, as well as promoting neuroprotective signalling through the MAPK/ERK pathway (Soeda et al. 2008). In addition, PAI-1 has been shown to promote microglial cells migration and to modulate their phagocytic activities (Jeon et al. 2012). However, PAI-1 has also been linked to several ND including AD, PD and MS, but never to ALS (Gverić et al. 2003; Yates et al. 2017; Reuland and Church 2020). In fact in MS, increased PAI-1 levels conduct to incapacities of the tPA/plasmin system to clear fibrin deposits and thus contribute to axonal damage; similar mechanisms were reported in AD with decreased clearance and degradation of amyloid- β (Gverić et al. 2003; Yates et al. 2017). Inhibition of PAI-1 in AD mice presented lower levels of plasma and brain amyloid- β and reversed cognitive deficits (Jacobsen et al. 2008; Angelucci et al. 2019).

In our ALS iMP+iMNs and iMG+iMNs cultures the increased PAI-1 level could also present dual effects. We could speculate that in an early phase PAI-1 possibly promotes MNs survival via its anti-apoptotic activities (Kubala et al. 2018), however with time increased PAI-1 could lead to decreased phagocytic functions and thus accumulation of cellular debris which culminates with important inflammatory response and MNs cell death. In order to assess the role of mutant MNs towards the secretion of PAI-1 we realised mixed cultures with mutant ALS iMP and iMG cultured over control iMNs. The striking decrease of PAI-1 secretion in these conditions led us to suggest two hypothesis (i) either that the secretions was induced by mutant MNs as a “help me” signalling (ii) or that PAI-1 increased secretion was linked to innate immune cells intrinsic defects and that the presence of control MNs induced a “cooling down” signalling dampening PAI-1 secretion in mutant iMP and iMG. We will further validate these hypotheses by realising mixed cultures with control iMP or iMG cultures over mutant iMNs. If the signalling is mainly promoted from mutant MNs, this will raise the question as to whether this signal is harmful or beneficial for MNs survival, as PAI-1 seems to have both roles depending on the context in which it is secreted. The fact that

most of mutant cells presented this increase, one hypothesis is that there is here a common pathway between different ALS forms.

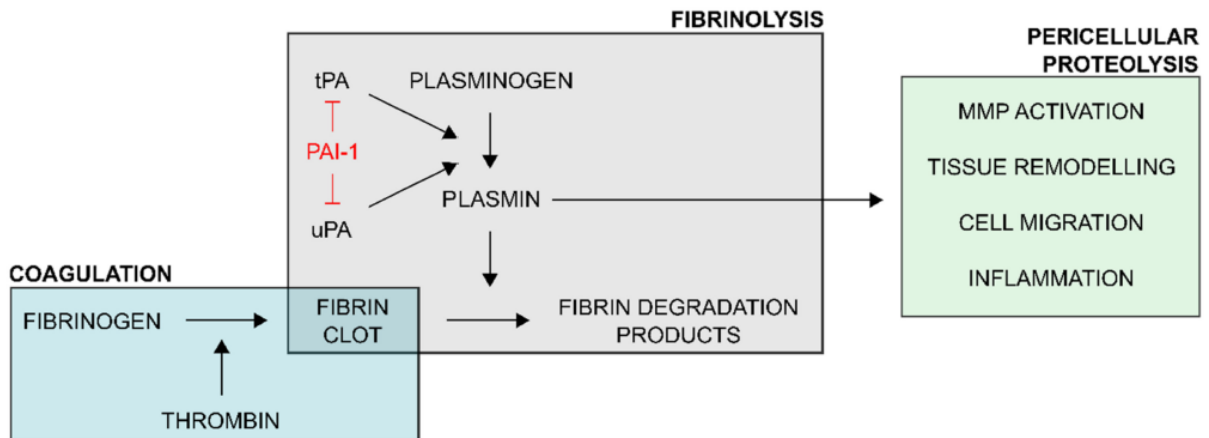


Figure 23 A schematic and simplified overview of the regulatory function of PAI-1 in the plasminogen activator / plasmin system. During the coagulation process, thrombin are formed which act on fibrinogen to form insoluble fibrin clots. In order to degrade these clots, the plasminogen activators (tissue type PA (tPA) and urokinase-type PA (uPA)) convert plasminogen to active plasmin form, which is a key enzyme responsible for the degradation of firbin clot. Through the uPA-mediated plasminogen activation plasmin extends its function to pericellular proteolytic processes including tissue remodelling, cell migration, adhesion and inflammation. PAI-1 oversees the plasminogen activator/ plasmin system by acting as an inhibitor of the plasminogen activators tPA and uPA thus inhibiting plasmin proteolytic activation.

PTX3 is part of the pentraxins family of multimeric proteins, which have important role in innate immune functions such as complement activation and opsonisation (Bottazzi et al. 2010). PTX3 was shown to be an acute phase biomarker. In normal condition the level of PTX3 is low and increases rapidly upon divers injury including endotoxic shock, sepsis as well as other inflammatory conditions (Azzurri et al. 2005; Wagenaar et al. 2009; Sprong et al. 2009). Studies in mice figured out that PTX3 has a host protective effect, although it could also exert negative effects under certain physiological conditions. Translational studies are now suggesting PTX3 as to be a useful biomarker for inflammatory diseases, and could be suggested to be used in ALS (Jaillon et al. 2014). Interestingly we observed that the PTX3 secretion is mostly iMP specific and increased in mutants at the late time point of cultures. Similarly to the observation with PAI-1, we showed that the secretion could be modulated when mutant cells were cultured over control MNs. PTX3 being a marker of acute inflammatory response, this could suggest that over time in culture, ALS MNs start to demonstrate increasing suffering which conduct to the activation of the innate immune cells and conduct to important release of PTX3. Similarly to PAI-1, mixed cultures with control iMP over

mutant iMNs will be important to understand the role of mutant iMNs on PTX3 secretion and to delineate if the increased level of PTX3 in mutant iMP may also be linked to internal defects linked to the mutation. PTX3 important increase could represent a transition phase from a more anti-inflammatory phase towards a more deleterious phase. In order to further, understand the role of PTX3 over time we will realise long-term cultures in order to assess if the level remain high over time or if the inflammatory profile evolve.

Overall, the identification of these two inflammatory factors have opened number of questioning as to their roles in MNs degeneration and their possibility as to be modulated to act on MNs survival. In order to determine if these responses are rather beneficial or deleterious we will conduct mixed cultures with controls iMP and iMG over mutant iMNs, as well as modulating the level of these factors in our cultures in order to see if we can bring trophic support to ALS MNs. Taking one step back, to date, although several studies proposed inflammatory biomarkers in ALS no consensus exists regarding the use of these markers in routine (Blasco et al. 2017; Gille et al. 2019; Swash 2020). PTX3 and PAI-1 being shared markers between the different ALS mutations, they could represent interesting factors to be measured in ALS patients to monitor inflammatory responses. Additionally, PTX3 seems to be mostly iMP specific in our cultures, thus it could serve as a marker mostly specific to peripheral inflammation. In fact, to date the development of immune modulatory drugs for ALS has been hampered by the absence of surrogate markers of therapy's response in clinical trials. In the past years, imaging techniques have emerged, as possible biomarkers to monitor inflammatory responses in the brain, yet circulating factors could be complementary to the practice of imaging which remains a cumbersome in use.

2.2.4 Limitations of our system and further perspectives

Although our experiments bring interesting results, to better understand the reaction of innate immune cells in the context of MNs degeneration *in vitro*, it is undisputable that the system remains reductive and cannot simply be extrapolated to the complex system that is the body. Nevertheless, it makes it possible to identify leads, which can further be studied *in vivo* in ALS patients and animal models.

As discussed in the microfluidic chapter, our iMP+iMNs co-culture conditions are not optimum as we cannot preclude an effect of iMP contact with soma and subsequent activations, which would not completely recapitulate the in vivo microenvironment. In order to better respond to our problematic these experiments will be reproduced in microfluidic devices.

Lastly, our cultures have offered an insight in the temporality of secretion profiles in ALS mutant iMP and iMG overtime and we showed that it was possible to model an evolution from an anti-inflammatory context to a less clearly defined inflammatory state in co-cultres with mutant MNs. However, we also noticed that at the two time points of our analysis MNs did not seem to degenerate rapidly enough to induce a switch between a protective to a deleterious responses from iMP and iMG. This being, we observed for some mutant cells that few days later (after day 11), cultures were so stressed that it was too difficult to analyse individual cells in co-cultures. Analysis of cultures maintained longer in cultures have now to be carefully considered. For this purpose, I have already collected supernatants of later time points for iMG+iMNs cultures. Inflammatory factors will be measured in these samples, with the hypothesis that we will measure more pro-inflammatory factors in these cultures attesting of the MN suffering and of the switch of inflammatory cells from a beneficial to a detrimental reaction.

Taken together, I think that our study paints a picture of a temporality of inflammatory factor secretions over time in ALS iMP and iMG in contact with ALS iMNs in culture. Interestingly, we report different profiles of secretion between iMPs and iMGs, as well as between the different ALS mutants. While some inflammatory factors were not detected in our culture system, some others were part of a “classical” inflammatory response shared by control and ALS iMP and iMG, yet some factors appeared more specific to one mutant or another in iMG and iMP. Lastly, the identification of PAI-1 and PTX3 as two inflammatory proteins induced at the late time point and being specific of ALS innate immune cells, represent interesting factors for further exploration in ALS animal models and patients.

More globally, the better understanding of the temporality of inflammatory events between ALS subtypes and in the CNS and PNS would help to assess the different adjustable time windows to act on inflammation and possibly the survival of neuronal cells.

3- Alteration of the cellular degradation machinery: a shared pathomechanism between ALS microglia and macrophages

Proteostasis impairment in ALS has been increasingly studied in the past years as detailed in the introduction (Ruegsegger and Saxena 2016). Studies have also demonstrated that protein aggregates can interact and disturb proteostasis (Soo et al. 2015). The identification of numerous ALS linked genes which are interacting directly or indirectly with the degradation machinery pathways have strengthened the interest of the pathway as a possible commonly altered cellular mechanism amongst different ALS subtypes (J. O. Johnson et al. 2010; H.-X. Deng et al. 2011; Fecto et al. 2011; Freischmidt, Müller, et al. 2015). In ALS patient's post-mortem tissues, TDP-43 aggregates are found in more than 95% ALS cases, suggesting that even in ALS patients without any identified genetic cause, the presence of these aggregates could contribute to the impairment of proteostasis.

Most of the studies have to date focussed on the alteration of proteostasis in MNs as they are the primary targeted cells in ALS pathology. However, the importance of non-neuronal cells in the progression of the disease is now well admitted. ALS genes being ubiquitously expressed, with some genes being even more linked to immune functions (TBK1, C9orf72), proteostasis disturbances might also occur in these cells including immune cells and represent a vulnerable point which could have major importance in ALS pathogenesis.

3.1 Importance of proteostasis in innate immune cells

Phagocytes from the innate immune system are characterised for their important clearance functions which contribute to the maintenance of tissue homeostasis in vivo. In vitro, it has been shown that phagocytes are also able to induce their phagocytic capacities when adding synthetic compounds mimicking PAMPs and DAMPs (Hamczyk, Villa-Bellosta, and Andrés 2015). Upon phagocytosis, the cargo will transit between different pathways which can include the UPS or autophagy systems (detailed in the introduction Chapter 3 3.1.2.2 and the Chapter 5). In the UPS the target is degraded by the proteasome while in the autophagy system the cargo culminates in the lysosomal pathway which degrades and recycles some of the components.

Innate immune cells are highly active cells, which necessitate important turn over and solicit the degradation machinery. Additionally, in the past years, a growing body of evidences suggested a bidirectional association between protein and quality control system and inflammation (Su et al. 2016). In fact, aging has been shown to be associated with disrupted UPR activation in animal models leading to neurodegeneration, although the underlying mechanism governing this neurodegeneration remains not well described (Paz Gavilán et al. 2006; Naidoo et al. 2008; Gavilán et al. 2009; Salminen and Kaarniranta 2010; R. C. Taylor 2016). Moreover, in aging individuals it has been reported an increased chronic inflammation demonstrated by higher levels of cytokines, chemokines and acute phase proteins circulating in the biofluids as well as increased expressions of genes involved in inflammation (Kotas and Medzhitov 2015; Franceschi et al. 2017; Furman et al. 2017; J. M. Bennett et al. 2018; Ferrucci and Fabbri 2018). This phenomenon has been named inflammageing and is thought to increase susceptibility to develop certain age related diseases including AD, PD, ALS, cardiovascular diseases, diabetes and cancer. In vitro and in vivo, it has been showed that LPS-induced neuroinflammation conducted to ER stress, however inversely the UPR activation has been found to increase the production of inflammatory cytokines. Thus suggesting that inflammation and proteostasis are intertwined, making it hard to fathom who is the chicken and who is the egg in the story.

In ALS, evidence of altered proteostasis in innate immune cells has been shown in C9orf72^{(G4C2)ⁿ}. Bone marrow derived macrophages and microglia from C9orf72^{(G4C2)ⁿ} knockout mice demonstrated increased lysotracker and Lamp1 staining suggesting defects in the late endolysosomal trafficking. The early endosomal trafficking did not show impairment in this study (stained using Rab5 and Rab7). Lamp1 accumulations was also observed in C9orf72^{(G4C2)ⁿ} post mortem's tissues. In adjunction, the authors reported increased IL-10 (in bone marrow derived macrophages only of C9orf72^{(G4C2)ⁿ} mice), IL-6 and IL-1 β .

3.2 Altered endo-lysosomal pathway in ALS mutant iMP and iMG

To our knowledge, our study is the first one comparing endo-lysosomal pathway functionalities in ALS iMP and iMG using a similar experimental approach and in a human context.

3.2.1 Late endo-lysosomal compartment

The late lysosomal compartment is the culminating step of endosomal, phagocytic and autophagic pathways. Lysosomes are membrane bound compartments containing digestive enzymes with functions to recycle damaged organelles as well as nucleic acids, polysaccharides, fats and proteins. In the past years, the knowledge regarding biology has largely evolved and lysosomes are now viewed as highly dynamic organelles involved in multiple of cellular functions including signalling, immunity, adhesion, migration as well as capable of responding to microenvironmental stimuli (Ballabio and Bonifacino 2020, Saffi and Botelho 2019). Dysfunction in lysosomal compartments and functions have been associated with number of pathologies including inflammation cancer, ND, and specific lysosomal stage diseases.

In our study, we used LysoTracker, which is a commercial fluorescent acidotropic probe, which label acidic compartments and lysosomes. In our co-cultures conditions, we observed increased volumes of acidic compartments in C9orf72^{(G4C2)ⁿ} iMP similarly to the observations in C9orf72^{(G4C2)ⁿ} knockout bone marrow derived MP (ORourke et al. 2016). We further confirmed the observations using Lamp1 staining, the results are summarised in the Table 12. Interestingly, while the individual Lamp1+ vesicle volumes were increased in C9orf72^{(G4C2)ⁿ} iMP, the volumes were decreased in SOD1^{N139D} and TARDBP^{G348C} iMP when compared to controls (Table 12). Based on these observations, we wondered if this could conduct to impaired lysosomal dynamics. In fact, some preliminary results conducted on different mutant iMP support an altered intracellular dynamic in mutant iMP compared to controls. Interestingly, the presence of very large structures in C9orf72^{(G4C2)ⁿ} iMP appeared to engineer steric hindrance in cells preventing normal organelles dynamics. The decreased motility was also observed in SOD1 and TDP-43 mutants. Experiments need to be repeated and further studies would be required to understand if the increased volumes and dynamics in the structures are able to prevent the lysosomal compartment dynamics, thus leading to subsequent alterations of proteostasis and other defects.

The increased lysosomal volumes observed in C9orf72^{(G4C2)ⁿ} iMP was also thought to be linked to potential important “over-phagocytic” reactions of mutant iMP compared to controls, possibly linked to more suffering of mutant iMNs in culture. However, when we cultured the C9orf72^{(G4C2)ⁿ} iMP over control iMNs we still observed increased lysosome volumes. These observations suggest

that the alteration of lysosomes seems to be intrinsic to C9orf72^{(G4C2)ⁿ} iMP and could reflect defects in fusion/fission with vesicles containing cargo or other lysosomal defects leading to accumulation of undegraded materials. On the other hand, the decreased volumes in other mutants may be linked to an overloading of the early endosomal system (which will be discussed below), or a defective lysosomal pathway leading to compensatory mechanisms (discussed below).

Table 12 Summary of the observed endo-lysosomal alterations in ALS iMP cultured over ALS iMNs.

| iMP+iMN | Number of vesicles | Mean volume per cell | Individual ves volume |
|---------|---|---|---|
| Lamp1 | TARDBP ^{G348C} ↗** SOD1 ^{N139D} = mC9orf72 = | mC9orf72 ↗** SOD1 ^{N139D} ↘*** TARDBP ^{G348C} = | mC9orf72 ↗*** SOD1 ^{N139D} ↘**** TARDBP ^{G348C} ↘**** |
| EEA1 | mC9orf72 ↗*** SOD1 ^{N139D} = TARDBP ^{G348C} = | mC9orf72 ↗*** SOD1 ^{N139D} ↗**** TARDBP ^{G348C} ↗**** | TARDBP ^{G348C} ↘*** SOD1 ^{N139D} = mC9orf72 = |

In iMG the volumes of lysosomes were smaller in all mutants compared to controls (results are summarized in Table 13). Nonetheless, the neuronal network was deprived of major cellular debris suggesting that mutant iMG were able to clear cellular debris and that the degradation machinery was in fact functional. It has been suggested that under some conditions, compensatory mechanisms can occur between different degradation pathways. Some evidences showed that autophagy dysfunction can lead to proteasome compensation (Ruano 2021). For instance in in vitro cultures of human colon cancer cells, autophagy impairment by RNA interference of autophagy related genes led to upregulated transcriptional expression of proteasomal subunits, as well as proteasomal activities (X. J. Wang et al. 2013). This compensatory mechanism was also observed in vivo in mice with defective chaperone mediated autophagy, increased content of the 19S proteasomal regulatory particle demonstrating that basal proteostasis was compensated by proteasomal activity. Additionally in these mice, it was shown that the compensatory mechanisms were efficient in young mice but became not sufficient against proteotoxicity linked to aging or induced by stress (Schneider et al. 2015). In fact, we could speculate that in our culture conditions, mutant iMG have defective autophagy system and thus compensate via other degradation

pathways. In order to investigate this hypothesis it would be interesting to block the proteasome in our cultures, by for instance the use of MG132, as well as possibly staining our cells with other markers, which are implicated in other degradation pathways such as ubiquitin and p62. Additionally, some probes have been designed to measure the proteasome activity. It would be particularly interesting to assess its activity in our cell cultures at different culture time points in order to see if over time some compensatory mechanisms occurred (Gan et al. 2019). In relation to the study from Schneider et al. the compensatory mechanism seemed to be efficient in young mice, but not when cellular stress occurred (Schneider et al. 2015). Paralleled with our study, we could imagine that at this stage of the culture the compensatory mechanism may still be functional but over longer period of culture with MN degeneration the pattern may differ. We have now to reproduce these analyses at earlier culture time points and later culture time points, to have a better overview of the dynamic and cross talk between these different pathways.

Table 13 Summary of the observed endo-lysosomal alterations in ALS iMG cultured over ALS iMNs.

| iMG+iMN | Number of vesicles | Mean volume per cell | Individual ves volume |
|---------|--|---|---|
| Lamp1 | mC9orf72 ↓ *** SOD1 ^{N139D} ↓ **** TARDBP ^{G348C} ↓ **** | mC9orf72 ↓ **** SOD1 ^{N139D} ↓ **** TARDBP ^{G348C} ↓ **** | mC9orf72 ↓ **** SOD1 ^{N139D} ↓ **** TARDBP ^{G348C} ↓ **** |
| EEA1 | mC9orf72 = SOD1 ^{N139D} = TARDBP ^{G348C} = | mC9orf72 = SOD1 ^{N139D} = TARDBP ^{G348C} = | mC9orf72 ↓ **** SOD1 ^{N139D} ↓ **** TARDBP ^{G348C} ↓ **** |

Taken together, interestingly we have observed different types of alterations in the volumes of lysosomes between iMP and iMG as well as between the different ALS mutations. Several hypotheses support a defect in transport routes in C9orf72 mutants cells supporting our observations: (i) C9orf72 was shown to be associate with Rab1 thus and may modulates ER-Golgi transport (Farg et al. 2014), (ii) C9orf72 knock down impairs endocytic trafficking from the plasma membrane to the Golgi apparatus (Y. Aoki et al. 2017), (iii) C9orf72 was shown to interact with Rab11 endosomes and to act as a GEF for Rab8 at the membrane (Farg et al. 2014; Sellier et al. 2016b). In other ALS forms, it is known that aggregated forms of mutant the protein SOD1 or TDP-43 can directly interact with degradation pathways, thus impairing their normal functioning. This

could somehow explain the diminished volume of lysosomes we observed in our cultures. However, we could also speculate that this decreased volume is linked to other defects in lysosomal pathway, leading to compensatory mechanisms via other degradation routes including proteasomal system. It will be interesting to stain the cells with antibodies against aggregated proteins in conjunctions with some markers of the degradation machinery, in order to see if some direct links exist between the two.

In other ND, alteration of the lysosomal pathway has also been reported but mostly in neuronal cells. For instance in AD, defects in endolysosomal transport resulted in alteration of amyloid- β production (Nixon 2017), while in Huntington the huntingtin aggregates inhibited autophagosome biogenesis and transport (Martinez-Vicente et al. 2010; Wong and Holzbaur 2014; Rui et al. 2015). Disruption in degradation pathways could be a common pathological mechanism between different ND, which may not have the same origin (i.e. different aggregates forms) but culminating with increased cellular stress fuelling neuronal degeneration.

3.2.2 Early endo-lysosomal compartment

Early endosomes are characterised by the initial vesicles formed during endocytosis. Early endosomes serve as hub for sorting internalised and plasma membrane associated cargoes that will be recycled to the plasma, transported to or from the Golgi apparatus directed to lysosomes for degradation (Pier Paolo Di Fiore and von Zastrow 2014). Interestingly, endosomes display at their surface signalling platforms that transduce external and membrane receptor signals via activation of intracellular signalling molecules (Cosker and Segal 2014; Cendrowski, Mamińska, and Miaczynska 2016). Defects in early endosomes have been reported in several diseases including AD and Pick Disease type C (Nixon 2005; Maxfield 2014).

In iMP+iMNs ALS mutant cultures, we have observed an increased mean volume per cell of EEA1+ vesicles, however the individual vesicle volumes did not appear to be significantly different between mutants and was even decreased for TARDBP^{G348C} (Table 12). These results may be linked to a smoothing of the effect linked to the high heterogeneity of the volumes of EEA1 vesicles. The increase observed in EEA1 mean volumes was also observed in other ND (Nixon 2005; Maxfield 2014). The early endosome maturation process is complex, governed by numerous different factors and defects in maturation can lead to enlarged lysosomes. Several studies have

reported that modulation of different factors of the pathway may have direct effects on endosomes maturation and functions. Rab5 and Rab7 are GTPases governing the switch from early to late endosomes; defective Rab5 lead to enlarged early endosomes (Bucci et al. 1992; H. Stenmark et al. 1994; Wegner et al. 2010). In fact, C9orf72 was shown to colocalise with Rab5 which could support a role in early endosome maturation. Thus mutant C9orf72 may impair early endosome maturation and conduct to the increased early endosomal volumes observed in C9orf72 mutants iMP (Farg et al. 2014). Early endosomes increased volumes may also be linked to the ineffective downstream system as already described previously. In line with this, a recent study has shown that absence of autophagy can lead to aberrant early endosomes accumulation. In addition, endosomal homeostasis was shown to be maintained by autophagy. Lysosomes are recruited to damaged early endosomes requiring the activity of TBK1, Gal8 and the autophagy machinery. *TBK1* gene is found mutated in some fALS and sALS (Freischmidt, Wieland, et al. 2015; Freischmidt et al. 2017), reinforcing the idea that alteration of proteostasis may represent an important driver of ALS pathology. As we previously suggested that the diminished Lamp1+ vesicle volumes may be linked to defects in the machinery this may conduct to perturbation of early endocytic pathway (Fraser et al. 2019). Taken together, it seems that the increase of early endosome volumes is a shared pathological phenotype between ALS iMP however the reason of their enlargement and if this has a pathological relevance remains to be determined. Staining with other early endosomal markers such as Rab5 and late endosomal markers such as Rab7 could be interesting in order to see if the endocytic pathway is perturbed the whole way.

In contrast to the observations in iMP, ALS mutants in iMG displayed decreased EEA1+ vesicle volumes (Table 13). These differences may be due to dissimilarities in cellular dynamics. The neuronal cultures possibly demonstrated less suffering when they were cultured with iMG as iMG may displayed more anti-inflammatory environment due to the presence of TGF β 1 compared to cultures with iMP. Additionally the decreased volumes may also demonstrate less phagocytic and endocytic activities from these mutant iMG compared to control iMG, possibly linked to defects in the pathway. In fact co-cultures of mutant iMG on control iMNs albeit bringing trophic support, the cells seemed more toxic for neuronal network or possibly less trophic. These hypothesis need to be confirmed in future studies by realising longer timing of cultures. In fact, adding a cellular stressor in the culture such as LPS or other inflammatory polarisation molecules could stimulate

phagocytosis and we could monitor the endo-lysosomal pathway in mutant iMG under these conditions (Xue et al. 2014). Additionally, as was observed in iMP, possibly steric hindrances in iMG may lead to decreased intracellular trafficking and endolysosomal pathway functioning. Live imaging could help to confirm or infirm these hypotheses.

Overall, we have observed distinct profiles between iMG and iMP similarly to what was observed regarding the secretory profiles which reinforce our belief that the two cell types may have distinct roles depending on the ALS form. The better characterisation of these pathways in different ALS mutations as well as in sALS iPSC could help to identify commonalities and divergences, which could be modulated to act on neuronal survival. As a whole, our transcriptomic study supports our observations, as both inflammatory pathways as well as cellular degradation machinery were found to be significantly deregulated in mutant cells compared to controls. Interestingly, pathways linked to proteasomal degradation were also found to be significantly different in mutant iMG compared to controls supporting possible compensatory mechanisms, which would warrant further investigations.

3.3 Endolysosomal pathway link with inflammation

Inflammation and the endosomal pathway are highly linked by various mechanisms. Interestingly, a number of plasma membrane receptors can be regulated by endocytosis at different levels, notably cytokines receptors. Initially, internalisation of the activated receptors was considered as a way for signal attenuation, however it is now clear that endocytosis also regulates the duration of receptor signalling as well as specificity of signalling input and output (Barbieri, Di Fiore, and Sigismund 2016). IL-6 receptor was shown to undergo ligand independent constitutive internalisation, and IL-6 was shown to increase the rate of this internalisation and lysosomal degradation (Thiel et al. 1998; Cendrowski, Mamińska, and Miaczynska 2016). TNFR, IL-1R, IFNR are also endocytosed and could have different consequences in the cells.

As we have observed alterations of secretory profiles in iMP and iMG and disruption of the endo-lysosomal pathway, we could speculate that some of these observations are linked one to another and further investigations will be interesting in order to identify if they could represent possible targets to be modulated in order to act on neuronal survival. Some cytokines receptors

downstream signalling pathways involve cytosolic adaptors, which can be recruited at the plasma membrane but also on the intracellular endosomes. This can prolong the signalling event during receptor trafficking until the sequestration in intraluminal vesicles. Additionally some signalling pathways mediated from the same receptors could be both dependent or independent of the endocytosis processes. For instance for TNFR1, the ligand induction of the canonical Nf-κB pathway occurs at the membrane and do not depend on endocytosis inhibition; while in contrary the secondary signalling event which is the apoptosis and non-canonical Nf-κB pathway, is highly depends on the internalisation of the receptors and signal from endosomes (Schneider-Brachert et al. 2004; Tchikov et al. 2011; D'Alessio et al. 2010; Schneider-Brachert, Heigl, and Ehrenschwender 2013). In addition, receptor signalling can also reciprocally regulate the endocytic machinery (P. P. Di Fiore and De Camilli 2001; Pálffy, Reményi, and Korcsmáros 2012).

Other recent evidences linking the inflammatory system to the endolysosomal pathway come from McCauley et al. They showed that the loss of C9orf72 expression specifically in myeloid cells is sufficient to recapitulate the hyperophy and autoinflammation previously observed in C9orf72 knock out mice (ORourke et al. 2016; McCauley et al. 2020). Moreover, C9orf72 knockout bone marrow derived macrophages were selectively hyperresponsive to activators of Stimulator of interferon genes (STING), which is a key regulator of the innate immune response to cytosolic DNA. Interestingly, STING degradation through the autolysosomal pathway was diminished in these C9orf72 knockout in myeloid cells, and direct blocking of STING can reduce the hyperactive interferon response in C9orf72 knockout immune cells as well as the inflammation in whole body C9orf72 knockout mice (McCauley et al. 2020). These observations were validated in human tissues and seemed to correspond to a specific C9orf72 ^{(G4C2)ⁿ} patients' signature as they did not observe it in sALS. Although we did not observe increased IFN response in our culture, the link here establish between STING degradation and the autolysosomal pathway drive us to consider longer time cultures in order to assess if we would observe increase IFN responses.

Overall, endocytosis and signalling are closely and bidirectionally linked. However, the biology of signalling events underlying cytokines receptors endocytosis remain poorly understood and require further studies in order to better understand their importance in disease context (Hermanns et al. 2016).

3.4 Limitations and perspectives

Our study has permitted to give first evidences of defects in the endo-lysosomal pathway in ALS mutants iMP and iMG different from the C9orf72 in a human context. The different profiles we have observed between cell types support our previous observations with the secretory profiles and reinforce the idea that the two cell types may have distinct actions in ALS pathogenesis. We would further like to use microfluidic devices to better understand the respective contribution of macrophages and microglia on distinct cellular compartments and ultimately the synergic role they have when added in the respective compartment.

Additionally we have observed differences between mutant ALS and control cells. In order to better understand the temporality of the endo-lysosomal defects, earlier and later time points analysis will be interesting. This will permit us to appreciate if the defects are early events or accumulate with ALS MNs aging and increased axonal suffering.

To date we have focused our attention mostly on the endo-lysosomal compartment. The use of Lamp1 may be a limiting indicator of lysosomal biology, as it was shown not to be specific enough for studying lysosomes at least in neuronal cells. We sought to combine cathepsins D and B as well as glucocerebrosidase markers to better visualise lysosomes. Moreover, we would like to label our cells with different markers of the temporality of the endocytic pathway such as Rab5 and Rab7.

We can hypothesise that in our mutant cells some other degradative pathways may be also affected, or that the observations we made were linked to compensatory mechanisms by proteasomal or other systems. Thus, the possible modulation of the proteasomal functions could be interesting to measure differences compared to our current observations.

4- Reflect in vivo disease with more integrated cultures

The discovery of iPSC technology has of course first and foremost opened the doors to the modelling of the disease in a human context with an enormous diversity of variant mutations, as well as sporadic cases. Given that the MN is the “central” cell of the disease because it is this cell which when it degenerates is a reflection of progressive paralysis. Early studies from iPSC invariably focused on what conferred the vulnerability of MNs as well as the inherent flaws

associated with different mutations. Indeed, the first protocols to generate MNs arrived very quickly. Since then, protocols have emerged allowing the generation of astrocytes, oligodendrocytes, macrophages and microglial cells and others. Although these protocols are not yet all perfect in terms of yield, purity and temporality, they still offer the possibility of studying the pathology in new cell types. Since it is admitted that substantial non-cell autonomous effects are contributing to ALS (Ilieva et al., 2009), including from microglia (Boillée et al., 2006), astrocytes (K. Yamanaka et al., 2008), oligodendrocytes (Kang et al., 2013), and potentially muscle (Loeffler et al., 2016). This obviously raises the question of knowing what are the intrinsic defects of these surrounding cells which led them to become harmful in the environment, but also what is their role in neuronal well-being.

4.1 Co-cultures of non-neuronal cells with MNs

For this, different co-culture techniques have been implemented using human iPSC, mice primary cultures or even human post mortem derived cells. Several questions can be asked: whether the toxicity or topxicity is mediated by a soluble factor or if it necessitate direct contact? if the different ALS mutations confer the same beneficial or detrimental effect on MNs survival ? and additionally if the intrinsic defects in the non-neuronal cells are sufficient to cause control neuronal cells death or if it necessitate the mutation in the MNs as a “second hit” event?

Most of the studies to date have been done with astrocytes or oligodendrocytes. ALS astrocytes from familial and sporadic forms were shown to be toxic to control MNs survival when co-cultured directly with them (Hall et al. 2017; Smethurst et al. 2020; A. Zhao, Pan, and Cai 2020; Rajpurohit et al. 2020). Interestingly, even the conditioned media from ALS astrocytes is sufficient to induce MNs suffering showing that the toxicity may be partly mediated by soluble factors (Di Giorgio et al. 2007; Haidet-Phillips et al. 2011; Varcianna et al. 2019). To my knowledge, only one study used iPSC derived oligodendrocytes to study their role on MNs survival. In fact, oligodendrocytes from sporadic or familial ALS patients seemed to be both toxic towards MNs survival. Similarly to astrocytes, the conditioned media when replaced at 100% also conducted to increased MNs death within 4 days with the highest toxicity observed from SOD1 oligodendrocytes supernatant (Ferraiuolo et al. 2016). Regarding the contribution of microglia and macrophages in mice, only studies using primary microglia cells over control MNs have been conducted (W. Zhao et al. 2010; Thonhoff et al. 2011; Frakes et al. 2014). In fact a study demonstrated that SOD1^{G93A} microglia was

toxic for WT MNs survival via activation of the NF- κ B pathway and that blocking NF- κ B rescue MNs survival (Frakes et al. 2014). Conversely, constitutive activation of NF- κ B pathway in WT microglia can cause MNs death in vitro. Additionally, another study showed that extracellular SOD1^{G93A} and SOD1^{G85R} proteins could activate microglia, but that only extracellular SOD1^{G93A} activated microglial cells were toxic for MNs in vitro (W. Zhao et al. 2010). In our study we did not observe this important toxicity observed by previous studies, in fact we report a more trophic effect of microglia and macrophages towards MNs survival. However, the study design are not equivalent, and we may still be too early in the culture condition to reflect the possible toxicity. Additionally, the SOD1 mutant mouse model over expressed the human mutant variant which might enhanced the toxicity. In fact in G93A SOD1 mice the CNS overexpression is of 17 fold compared to WT mice, while G85R is 0.2-1 fold (Philips and Rothstein 2015). The fact that the toxicity towards MNs was not observed with SOD1^{G85R} microglia in the study from Zhao et al. may reflect more closely the patient condition.

Thus, our study is the first one to date to study and compare in a same experimental context the toxicity and trophicity of human ALS microglia towards matched ALS MNs. Additionally, no previous studies have looked at ALS macrophages role on MNs survival.

Mismatched and matched co-cultures are particularly interesting to identify the origin of neurotoxicity and could better identify the cell type that has to be targeted and in which phase of the pathology. Further co-cultures with various other cell types and with also iPSC derived MNs would be warranted to better identify the shared or mutation/cell type specific pathological mechanisms. In our study, we realised mixed co-cultures and observed that most of the defects were still present in mutant iMG and iMP. However, we identified a significant decrease of PTX3 and PAI-1 secretion when iMP were cultured over control iMNs suggesting that the environment might contribute to the secretion of these two factors. We would like to realised other mixed co-cultures using control iMG and iMP over ALS mutant iMN in order to measure the contribution of ALS MNs on iMP and iMG activation. This would permit to better identify some possible modulatory mechanisms specific to the different cell types that are innate immune cells or MNs.

4.2 The complexity of mimicking the immune system in vitro

The immune systems are composed of a variety of hematopoietic cell types which are involved in host defense responses as well as a variety of other supportive functions. In vitro new recent

protocols to generate iPSC derived innate immune cells including, mast cells, APC and NK (Kovarova et al. 2010; Igarashi et al. 2018; Kauts et al. 2018; Ikuno, Ito, and Inoue 2019; Ackermann, Dragon, and Lachmann 2020; G. Bian et al. 2021; Shankar, Capitini, and Saha 2020), offer new perspectives as to study the role of other innate immune cells in various diseases. On the other side, the generation of iPSC derived adaptive immune cells is more challenging and to date only immature B and T cells can be obtained (French et al. 2015; Böiers et al. 2018; Montel-Hagen et al. 2019; Richardson et al. 2021).

Owing to the complexity of the immune systems and the numerous interactions required between different cell types in order to mediate their biological functions, we imagine that cultures with more cell types could better mimic in vivo environment. Indeed, we have herein used macrophages and microglia with MNs culture and have observed interesting first results, nonetheless we are conscious that in vivo macrophages and microglia are interacting and reacting with a number of other cell types and circulating factors contributing to their polarisation and governing the underlying response. In these two cell types cultures, we sought that external activator for microglia and macrophages may be lacking in order to better reflect the in vivo disease. Several options could be interesting to remedy to these limitations: (i) activation of the immune system with synthetic compounds mimicking a state of activation such as using a cocktail of specific cytokines (Xue et al. 2014), (ii) protein aggregate induced formation in mutant cells to prime macrophages and microglia and conduct to more pronounced responses towards MNs suffering, (iii) generation of tri or tetra cultures with iPSC derived innate immune cells or adaptive cells, which could possibly mature in contact with iMG and iMP. This last option is particularly interesting, as it would allow seeing the role of other immune cells in ALS pathogenesis. In fact, the use of iPSC with the same mutant genetic background might help to better understand the role of various non-neuronal cells in ALS pathology. Further on by realising mixed cultures with the different cell types could permit to see if by modulating one or another cell type one can act on neuronal survival.

Overall, iPSC technology offers a myriad of possibilities. The recent appearance of new protocols allowing the generation of many immune cell types, glial cells, muscle cells and many neuronal subtypes, opens new avenues for the development of more complex in vitro models which could

recapitulate in vivo patient's disease. With a disorder like ALS, introducing mutations in wild-type iPSC could also be of interest to decipher which part of the pathological defects is directly linked to the mutation and which part depends on the environment. Additionally, in the past years iPSC technologies has proven efficacy for drug screening. Although most of the current trials have been done on neurons, further studies using other cell types as well as co or tri-mutant cultures would be particularly interesting in the context of multifactorial diseases, including ALS.

General conclusion

The aim of my work was to study the respective role of human macrophages and microglia towards ALS MNs survival in different ALS forms.

We have settled a protocol allowing us the generation of iPSC derived macrophages and microglia bearing distinct transcriptomic signatures. In line with previous studies, we shed light on the importance of the microenvironment for proper cell differentiation and maturation. Next, as a proof of concept, we have realised mixed and matched co-cultures between ALS mutant iMP and iMG on ALS and control iMN. We identified cell type specific as well as mutations specific signatures, which were confirmed by our transcriptomic analysis. The secretome as well as the endo-lysosomal pathway appeared to be a shared deregulated phenotype in mutant cells, although the deregulation appeared to be mostly mutation specific. These observations support the hypothesis that proteostasis impairment may be a central pathomechanism in ALS pathogenesis, not only in neuronal cells but also in other non-neuronal cells such as macrophages and microglia. Additionally, the cell type specific reaction we observed supports the hypothesis that macrophages and microglia may contribute differentially in ALS pathogenesis and should be modulated distinctly across the disease (Chiot et al. 2020). In order to confirm these differences in cell type specificity and to mimic as closely as possible the respective microglia and macrophages micro-environments in vivo, we have developed, in collaboration with Dr Jean-Michel Peyrin, specific microfluidic devices which allow strict separations between MN cell soma and axons within two distinct chambers in which macrophages and microglia can be seeded. Specific designs prevent the migration of macrophages towards cell soma. This model will be particularly interesting in order to understand the respective, but also synergic contribution of ALS macrophages and microglia towards ALS MNs survival. In the next step of this project we will go a

step further using these microfluidic devices to realise co- and tri cultures. Based on our ALS iMG and iMP transcriptomic analysis we seek to identify mutation specific pathways deregulated as well as target genes, which could be modulated in “compartment specific manner” (either at the axonal part or soma part) in microfluidic devices in order to act on MNs long term survival.

On a larger scale, using iPSC of other fALS and sALS patients, we hope being able to perform a broader screening to identify common and distinct inflammatory signatures that will allow us to better classify the different subtypes of ALS. With ambitions, this leaves us to imagine the possibility of identifying time windows as well as pathways that could be modulated in a specific way for the different sub classifications of ALS phenotypes and act on disease progression.

APPENDICES

Appendice 1

Review submitted and currently in revision in the journal *Frontiers in Molecular Neuroscience*, section Brain Disease Mechanisms.

Human Induced Pluripotent Stem cells to study neuroinflammation in ALS and FTD 1

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Keywords: ALS, FTD, inflammation, immune system, iPSc, immune modulatory molecules

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ABSTRACT

Inflammation is a shared hallmark between ALS and FTD. For long, studies were conducted on tissues of post-mortem patients and neuroinflammation was thought to solely be the consequence of the disease with the immune system reacting to dying neurons. In the last two decades, thanks to improving technologies, the identification of new genes and the development of new tools and models, the implication of inflammation as a potential driver of the disease has emerged and evolved as a new area of intense research.

In this review, we will first summarize what is known in both ALS and FTD regarding neuroinflammation in patients, animal and cellular models and we will discuss the reasons of failures linked to therapeutic trials with neuromodulator drugs. We will then present the iPSc technology and its interest as a new tool to have a better immunopathological comprehension of familial and sporadic forms in a human context. The iPSc technology giving the unique opportunity to study cells across differentiation and maturation times, brings the hope to shed light on the different mechanisms linking neurodegeneration and activation of the immune system. Studies of monocultures of iPS-derived immune cells, of co-cultures with neurons and on 3D cultures, as more integrated cellular approaches, with different cell types (brain organoids), human iPS-derived cells will for sure allow to identify disease-specific defects in the different cell types and to decipher the synergic effects between neurons and immune cells. These new cellular tools could help to find new therapeutic approaches for ALS and FTD.

51 INTRODUCTION

52
53 Inflammation is a shared pathological hallmark of many neurodegenerative diseases. The
54 physiological function of inflammation is to defend our organism against various insults
55 implying a myriad of different cell types and molecular pathways. In many neurodegenerative
56 diseases, insults may come from the different disease-affected cells that can degenerate and die
57 or can secrete abnormal proteins that become immunogenic. Today, it is well documented that
58 inflammation is not a secondary event in the course of neurodegenerative diseases and that its
59 modulation could be of interest in therapeutic perspectives. Inflammation modulation would be
60 particularly interesting for all sporadic cases whom represent most of the patients affected by
61 neurodegenerative diseases. Modulating the inflammatory response could then be a strategy to
62 rapidly slow disease progression.

63 This review focuses on Amyotrophic Lateral Sclerosis (ALS) and Frontotemporal Dementia
64 (FTD), two neurodegenerative diseases in which overlap clinical presentations, pathological
65 mechanisms and genetics. The current knowledge about inflammation in patients, new
66 hypotheses brought by animal studies and the current state of clinical trials targeting
67 inflammation will be presented. Next, will be discussed involvements of both innate and
68 adaptive immunities and the temporality of inflammatory events. This temporality could be a
69 key to identify a time window that could be precisely targeted to twist the immune system in
70 the right direction and stop disease evolution. With a detailed picture of inflammatory events
71 in ALS and ALS-FTD, the current possibilities offered by the induced pluripotent stem cell
72 (iPSc) technology to generate different human immune cell types and to study their intrinsic
73 defects will be described. With the emergence of more integrated cellular approaches with
74 different iPS-derived cell types, interactions and synergic effects between immune cells and
75 neurons could be deciphered and bring new insights for innovative therapeutic approaches for
76 ALS and FTD.

79 INFLAMMATION IN ALS AND FTD

80
81 ALS and FTD disorders are two ends of a spectrum of neurodegenerative diseases. ALS clinical
82 presentation is characterised by progressive paralysis of voluntary muscles due to loss of both
83 upper and lower motor neurons, leading in most cases to the death of patients by respiratory
84 failure. ALS shares clinical and pathological features with FTD, a type of dementia
85 characterised by impaired judgment and executive skills. In FTD, the loss of cortical motor
86 neurons is accompanied by loss of neurons in the frontal and temporal cortices, which correlates
87 clinically with the symptoms of FTD (Neumann et al., 2006; Burrell et al., 2016).

88 Whereas survival is 3 to 5 years for ALS patients, life expectancy in FTD is less clear. Up to
89 50% of ALS patients develop frontal lobe dysfunction or language impairment. In FTD, some
90 studies claim only 5-10% of FTD patients with ALS signs (Rosso et al., 2003; Goldman et al.,
91 2005; Johnson et al., 2005; Seelaar et al., 2007), while other studies report around 50% of FTD
92 cases with subclinical motor neuron degeneration (Lipton et al., 2004; Mackenzie and Feldman,
93 2005). These differences are certainly due to variability in disease definitions and assessments
94 by clinicians. Some more recent studies indicated that FTD-ALS has a particularly poor
95 prognosis with a survival of 2-5 years (Kansal et al., 2016).

96 At the genetic level, several genes with numerous pathogenic variants, are causal for ALS,
97 ALS-FTD and FTD (Belzil et al., 2016). The same genetic mutation can result in either ALS,
98 FTD or both, implying a role - still badly understood - of other specific disease modifiers. Also,
99 as most mutated genes encode ubiquitously expressed proteins, they may affect differentially
100 various cell types contributing to the complexity of the disease (Ferrari et al., 2011).

101 Interestingly, the protein atlas shows that some genes linked to ALS and FTD are more
102 expressed in non-neuronal cells. Of particular interest are C9orf72 and PGRN genes encoding
103 C9ORF72 and progranulin proteins (Greaves and Rohrer, 2019). Hexanucleotide repeat
104 expansions in C9orf72 have been identified in up to 40% of familial ALS (fALS) patients, and
105 20% of familial FTD patients, in addition to around 6% of sporadic ALS (sALS) and FTD
106 patients (DeJesus-Hernandez et al., 2011; Renton et al., 2011). Mutations in the progranulin
107 gene (GRN) and the microtubule-associated protein tau (MAPT) have been identified as a major
108 cause of familial FTD (Greaves and Rohrer, 2019). Moreover, recent studies shed light on the
109 role of ALS and FTD genes implicated in innate immune related functions (TBK1, OPTN,
110 SQSTM1) (Abramzon et al., 2020) warranting the importance of the contribution of non-
111 neuronal cells in the pathology.

112
113

114 **INFLAMMATION IN ALS AND FTD: A SHARED PATHOLOGICAL HALLMARK**

115

116 **First Observations in Post-mortem Tissues of Patients**

117

118 Post-mortem studies brought the earliest observations assessing the presence of inflammatory
119 signs in ALS and FTD patients. At that time, no gene was identified to be linked to ALS or
120 FTD pathologies. Numerous post-mortem studies revealed the implication of several cell types
121 (different from affected motor neurons) in these pathologies.

122 Several ALS post-mortem case reports described decreased number of Betz cells in the cerebral
123 cortex and of motor neurons in the spinal cord accompanied by increased microglial activation
124 and astrogliosis (Brownell et al., 1970; McGeer et al., 1993; Nihei et al., 1993; Schiffer et al.,
125 1996; Saberi et al., 2015). Interestingly sensory neurons of the spinal cord dorsal horns were
126 not presenting increased microgliosis, strongly suggesting the specificity of the latter in reaction
127 to degenerating neurons. More recent studies identified the presence of immature and activated
128 dendritic cells (DC) in ventral horns and corticospinal tracts of ALS patients (Henkel et al.,
129 2004) as well as the presence of activated CD68+ macrophages and of CD4+ and CD8+
130 lymphocytes in the vicinity of motor neurons (Kawamata et al., 1992; Henkel et al., 2004). At
131 the periphery, demyelination, axonal degeneration, abnormal motor end plates, axonal
132 sprouting and atrophic muscles were described (Bjornskov et al., 1984; Tandan and Bradley,
133 1985).

134 In FTD patients, symmetrical convolutional atrophy in frontal and anterior lobes were observed
135 (Ikeda, 2000; Tolnay and Probst, 2001; Bright et al., 2019). In the grey matter, microvacuolation
136 and gliosis in laminae I-III were seen in conjunction with neuron loss, while neurons of lamina
137 V were reported to be only mildly affected. Rare dystrophic neurites were described. In the
138 white matter, mild gliosis was observed in subcortical fibers and loss of myelin was sometimes
139 observed.

140 Taken together, these first observations allowed identification of inflammatory signatures in
141 ALS and FTD post-mortem tissues. Nevertheless, the latter represent a fixed time of the disease
142 which is usually in an advanced state, it is therefore difficult to conclude whether the
143 inflammation is an early or late event of the disease. The question of the temporality of the
144 inflammatory events remains unsolved but crucial, as inflammation can be beneficial or
145 detrimental for motor neurons depending on the disease evolution stage.

146

147 **Animal Models to Assess of the Role of Inflammation During the Course of the Disease**

148

149 While imaging techniques in patients were still at their infancy, the development of animal
150 models for ALS and FTD permitted studies of inflammatory events during the course of each

151 disease. Studies using the mutant SOD1 mouse models (the most commonly used models to
152 study ALS) revealed that human mutant SOD1 expression solely in neurons led to no or a very
153 tardive ALS phenotype (Pramatarova et al., 2001; Lino et al., 2002; Jaarsma et al., 2008),
154 suggesting that other non-neuronal cells are essential to drive an ALS pathology.
155 Complementary pioneer studies with the human mutant SOD1 gene specifically removed from
156 microglia and macrophages (Boillée et al., 2006) or from astrocytes (Yamanaka et al., 2008)
157 demonstrated increased life expectancy of the ALS mice, showing for the first time a crucial
158 role of surrounding cells and in particular cells of the immune system in disease progression.
159 Similarly, in a GRN deficient mouse model developing several neuropathological features of
160 FTD, increased astrogliosis and microgliosis were observed (Yin et al., 2009). When GRN was
161 specifically depleted in microglial cells, mice developed obsessive compulsive behaviors
162 suggesting a role of microglial cells in these aspects of the pathology (Krabbe et al., 2017).
163 Robust neuropathologic phenotypes characterizing FTD pathology appeared to be driven by
164 non-cell autonomous mechanisms, as Petkau et al. showed that specific KO of GRN in
165 microglial cells were not sufficient to recapitulate phenotypic defects (Petkau et al., 2017).
166 Therefore, these animal models allowed to assess that inflammation was a major player in the
167 pathology rather than being only a consequence of neuronal death.

168 **Human Studies to Decipher the Involvement of Inflammation in ALS and FTD**

169
170
171 In humans, studies were conducted at several levels including brain imaging studies and
172 biofluids analysis, helping to gather evidences on inflammation events in ALS and FTD during
173 the disease course.

174 Imaging studies are valuable tools to assess cerebral changes, spreading patterns, network-wise
175 propagations and inflammation. These tools are now even being investigated as potential
176 biomarkers for the identification of an ALS pheno-conversion corresponding to the transition
177 from a pre-symptomatic phase to a clinically manifest disease (Chew and Atassi, 2019; El
178 Mendili et al., 2019). Positron Emission Tomography (PET) is a functional imaging technique
179 using radioactive ligands to measure, amongst others, changes in metabolic processes. Different
180 radioligands targeting specific cellular substrates are available for different imaging purposes
181 depending on the cellular event which seek to be studied. TSPO, which corresponds to the 18kD
182 translocator protein, is highly expressed on activated microglia and astrocytes (Lavisse et al.,
183 2012; Betlazar et al., 2018) and helps to visualise inflammation and gliosis. Several generations
184 of TSPO radio-ligands succeeded which differed on their binding specificities. The latest in use
185 is [11C]-PBR28 which binds with an 80-fold higher specificity TSPO in comparison to the old
186 generation of radioligands (Kreisl et al., 2010). However recent studies put in light the presence
187 of a polymorphism affecting the TSPO binding affinity, assessing the importance to control this
188 factor in studies in heterogeneous populations of ALS or FTD patients. A new PET probe
189 [18F]CB251 was recently published and seems to be more specific for TSPO regardless of
190 polymorphisms (Kim et al., 2020). This new probe will be of particular interest for future
191 studies.

192 In vivo studies in ALS patients revealed an increased binding of [11C]-PBR28 only to motor
193 cortices in comparison to control subjects and positive correlations with the Upper Motor
194 Neuron Burden Scale (UMNB) and negative ones with ALSFRS-R scores (Zürcher et al., 2015;
195 Alshikho et al., 2016, 2018; Ratai et al., 2018). In FTD and ALS-FTD patients, increased TSPO
196 binding were observed in cortical frontal, mesial temporal, subcortical regions, prefrontal
197 cortex, hippocampal and para-hippocampal (Cagnin et al., 2004; Turner et al., 2004; Miyoshi
198 et al., 2010; Chew and Atassi, 2019). The overwhelming majority of imaging studies in patients
199 are cerebral. Despite numerous attempts to image the spinal cord in vivo (Bede et al., 2012)
200 technological constraints (i.e. respiration, cardiac movements, small cross-sectional area) have

201 precluded reliable quantitative spinal cord imaging in patients (El Mendili et al., 2019).
202 Amongst the scarce imaging studies assessing ALS patient's spinal cords, most of them used
203 magnetic resonance imaging techniques. Studies of metabolic changes in ALS patients' spinal
204 cords using whole body PET/computed tomography (CT) images are recent. Two independent
205 studies identified increased [18F]-FDG uptake in the spinal cord while it was decreased in the
206 brain motor cortex. [18F]-FDG is thought to reflect cell metabolism without specificity for glial
207 cells, but the observed hyper-metabolism in the spinal cord was suggested to represent an
208 increased inflammation and gliosis due to accumulating glial cells in reaction to degenerating
209 neuronal cells (Bauckneht et al., 2020). Further studies using specific radioligands will have to
210 confirm these observations. PET studies open new perspectives regarding our understanding of
211 spinal cord pathology in ALS patients and a step further in imaging studies is the possibility to
212 assess temporal changes during the disease course. Objectives are to obtain more precise
213 information on cell reactivity and spreading of inflammation and gliosis in ALS and FTD
214 patients' brains and spinal cords. To date in the largest longitudinal ALS PET study, 10 patients
215 underwent [11C]-PBR28 PET scans twice over a six months period, with a stable [11C]-PBR28
216 uptake over this period of time (Alshikho et al., 2018), suggesting a plateau of glial reactivity
217 shortly after symptoms onset. These first results are very encouraging and further longitudinal
218 studies would bring important data, especially if the same protocol can be proposed to
219 asymptomatic subjects in familial cases.

220 Taken together, imaging studies are particularly interesting as they offer insights in brain and
221 spinal cord pathological tissues at spatial and temporal levels, and bear hope for uses as
222 biomarkers. This being, imaging studies still hold limitations for studying inflammation in ALS
223 and FTD patients. As mentioned above, TSPO is also overexpressed in activated astrocytes
224 (Lavisse et al., 2012). Given the different roles of microglia and astrocytes in ALS and FTD
225 pathologies, it represents a significant limitation in data interpretations (Vivash and O'Brien,
226 2016). Thus, more specific radioligands targeting microglia or astrocytes have to be developed
227 in order to be able to draw strong conclusions regarding inflammation and gliosis in ALS and
228 FTD.

229

230 **Circulating Inflammatory Cytokines and Chemokines in ALS and FTD**

231

232 Presence of circulating inflammatory cytokines and chemokines in the blood and the
233 cerebrospinal fluid (CSF) are thoroughly studied in ALS patients and at a lesser extent in FTD
234 patients (Table 1). Recently, studies showed that in both ALS and FTD the pro-inflammatory
235 and multifunctional IL-6 cytokine was increased in patients in comparison to controls
236 (Galimberti et al., 2015; Gibbons et al., 2015; Ngo et al., 2015; Lu et al., 2016; Tortelli et al.,
237 2020). Furthermore, IL-6 was suggested to possibly correlate with disease progression in ALS
238 (Lu et al., 2016). Apart from IL-6, ALS and FTD patients have distinct cytokines and
239 chemokines circulatory profiles.

240 In ALS, a large panel of pro and anti-inflammatory cytokines and chemokines were described
241 as deregulated in patients compared to controls (Moreno-Martinez et al., 2019). However,
242 results are not always consistent amongst the different studies, probably because of the
243 heterogeneity of the studied population. This heterogeneity exists at different levels. (i) First,
244 in most studies patient's blood or CSF are analysed at one single time point corresponding to a
245 unique precise disease state (ALSFRS-score with large standard deviation). The inflammation
246 status of the different patients may thus be different when analysed, making the results hard to
247 interpret. The state of disease is most of the time measured with the ALSFRS scale, a scale that
248 does not necessarily reflect the pathological state at the cellular level. To date among the most
249 promising biomarkers for ALS are heavy and light chains neurofilaments, precisely measurable
250 in CSF and blood samples and being under scrutiny in many trials (Oeckl et al., 2016; Xu et al.,

251 2017; Benatar et al., 2018; Poesen and Van Damme, 2019). (ii) Second, some studies include
252 patients diagnosed with the El Escorial criteria and categorised as “possible”, “probable”, or
253 “definite” disease. This classification allows establishing if there is a correlation between the
254 diagnosis and the amount of circulating factors. However, some other studies take all ALS
255 cases as one single group of patients and do not take into account the diagnosis, making any
256 correlation impossible. (iii) Third, the site of onset of the pathology (spinal vs bulbar) may also
257 be a confounding factor if two groups are not considered separately. Indeed, progression of the
258 disease is very different between the two forms of the disease and so are probably the
259 inflammatory events over time. (iv) Next, most studies include sporadic cases or compare
260 familial and sporadic cases, which can’t be seen anymore today as homogeneous groups.
261 Knowing the importance of some mutated genes in the immune system, retrospective studies
262 may be interesting to compare sub-groups based on their genotypes. (v) Finally, CSF and blood
263 biofluids are distinct compartments as the CSF is part of the central nervous system (CNS)
264 while circulating molecules are in the blood. Both compartments may contain different types
265 and amounts of cytokines and chemokines depending on the state of the pathology.
266 Very few studies conducted longitudinal studies and no significant differences of cytokines and
267 chemokines secretions were observed between two times pathological points in patients (Saleh
268 et al., 2009; Ehrhart et al., 2015; Prado et al., 2018). As in most cases recruited patients were
269 already advanced in their pathology, a hypothesis is that if inflammation is an early event,
270 analysis was probably conducted too lately.

271 Amongst other factors found deregulated in ALS patients’ biofluids, immunoglobulins (IgG),
272 metabolic proteins, adipokines, iron-related proteins, oxidative stress markers were found
273 deregulated in patients in comparison to controls (Mitchell et al., 2010; Ngo et al., 2015; Blasco
274 et al., 2017). These deregulations reflect metabolic and inflammatory dysfunctions which may
275 be critical actors of pathogenesis. In vitro and in vivo studies demonstrated that ALS patients
276 IgG induced a selective motor neuron’s death, presumably involving calcium (Yi et al., 2000;
277 Obal et al., 2002; Pullen et al., 2004; Demestre et al., 2005). These studies support the role of
278 immunological mechanisms in the selective loss of motor neurons.

279 In FTD, most studied focus on CSF analysis. CSF in FTD patients revealed altered of
280 inflammatory chemokines and cytokines secretion with similarities to those found in ALS
281 patients, such as IP-10, TNF α (Sjögren et al., 2004), TGF β , CCL-2 (also called MCP-1)
282 (Galimberti et al., 2015), RANTES (Galimberti et al., 2008), IL-8 (Galimberti et al., 2006).
283 Some cytokines were also more specific to cognitive aspects of the pathology as they were
284 found increased in Alzheimer’ disease (AD) and FTD patients such as IL-11 (Galimberti et al.,
285 2008), IL-15 (Rentzos et al., 2006b) and some found decreased (IL-12) (Rentzos et al., 2006a).
286 This suggests possible similarities in pathologic mechanisms underlying FTD and AD. Besides
287 this, it seems that clinical cases with FTLT-DTP and FTLT-tau (2 different FTD forms)
288 pathologies harbor distinctions in some neuropeptides and chemokines secretions (IL-23, IL-
289 17) which could help to distinguish the two forms antemortem (Hu et al., 2010). Regarding
290 blood analysis, one study reported no statistical differences of FTD patients’ serum cytokines
291 compared to controls (Galimberti et al., 2015). In the blood, some lipid assays were conducted
292 and showed increase triglyceride levels in FTD patients, which correlated with body mass
293 index, while HDL cholesterol levels were negatively correlated with body mass index (BMI)
294 (Ahmed et al., 2014). More recently a lipidomic study focused more precisely on lipids implied
295 in 3 key aspects of FTD pathology: inflammatory processes, mitochondrial dysfunction and
296 oxidative stress (Phan et al., 2020). Amongst other findings, they revealed increased lipid
297 implicated in inflammatory responses, LPC and PAF (lysophosphatidylcholine and platelet-
298 activating factor) which are known as second messengers or immediate response molecules and
299 act on various immune targets (T lymphocytes, Microglia, Macrophages, neutrophils) (Chang
300 et al., 2003; Han et al., 2004; Scholz and Eder, 2017). In conjunction they found a decrease in

301 o-acyl-w-hydroxy fatty acids (OAHFA) which is known to exert anti-inflammatory effects. A
302 significant inverse correlation between LPC and OAHFA was measured supporting the
303 presence of increased inflammation in FTD patients.

304
305 Taken together, imaging studies and biofluids analysis showed that inflammatory events are
306 present in ALS and FTD patients along the disease. Having today a clearer insight in the
307 temporal inflammatory events in ALS and FTD in order to orientate properly the different
308 immunomodulating therapies would be of great interest.

309
310

311 **THE ROLE OF THE IMMUNE SYSTEM IN ALS AND FTD**

312
313 Inflammation is a multifaceted reaction implying different cell types and molecular pathways
314 (Figure 1). We will now describe the different cell types that are thought to play a role in
315 inflammatory mechanisms in ALS and FTD, and try to put in perspectives their specific
316 implications at different time points of the disease.

317

318 **Innate Immunity in ALS and FTD**

319

320 Innate immunity is the first line defense of the organism. It provides an immediate immune
321 response against a non-self pathogen and is conserved amongst vertebrates and invertebrates.
322 It encompasses a group of different cell types that bear different functions in order to facilitate
323 the clearance of external pathogens or recognise insults for the organism. These different cell
324 types may be also differentially distributed in the body, harboring specific functions depending
325 on their location and surrounding environment. Monocytes and granulocytes, that include
326 neutrophils, eosinophils and basophils, are circulating in blood whereas mast cells and
327 macrophages are located in tissues.

328

329 ***Phagocytes***

330 Amongst phagocytes different subpopulations emerge. Tissue resident macrophages are
331 heterogeneous populations differing on their embryonic origin and regarding their location in
332 tissues or niches in which they can acquire specific functions and distinct
333 profiles. Macrophages are antigen presenting cells (APC). The main role of APC is to process
334 antigens and present them on major histocompatibility complex (MHC) at cell surface. The
335 MHC-antigen complex is recognised by the T cell receptor (TCR), allowing T cell activation.
336 APC also express many co-stimulatory molecules that participate in T cell activation. APC
337 regroup several immune cell types in varied tissues, including dendritic cells, macrophages,
338 microglia, B cells and Langerhans cells (Kambayashi and Laufer, 2014). Macrophages are less
339 potent than dendritic cells but still enough to activate neighboring adaptive immune cells.
340 Macrophage activation states are multiple (Xue et al., 2014). Depending on the insult they
341 encounter, they can release a myriad of cytokines, chemokines and other factors to resolve
342 inflammation by activating neighboring cells and attracting cells at the site of injury. In
343 conjunction, their key phagocytic activities allow them to clean up cellular debris and thus cool
344 down local inflammation. Microglia are a specific subtype of macrophages present in the CNS
345 with a distinct embryonic origin and isolated from PNS (Ginhoux et al., 2010; Schulz et al.,
346 2012; Kierdorf et al., 2013; Gomez Perdiguero et al., 2015). Since few decades, neuroscientists
347 have tried to understand the role of microglia in the CNS. Microglia display extended
348 prolongations allowing them to patrol the whole brain parenchyma within a matter of hours,
349 making them very active cells in the CNS. Our current understanding of the exact roles of
350 microglia in healthy brains remains limited. While they play different known roles during brain

351 development, their functions in adults are thought to be identical to macrophages' generic
352 functions. In pathologic brains, microglia appear to be first line responders to neuronal
353 suffering, helping to clear cellular debris when neurodegeneration occurs.

354 The importance of macrophages and microglia in ALS and FTD has been well established;
355 however, as for very long no specific markers could differentiate the two cell types, especially
356 for *in vivo* studies in ALS models, both cell types were studied as a single population. In 2006,
357 two independent studies demonstrated that decreasing expression of human mutant SOD1 only
358 in macrophages/microglia in ALS mouse models led to slowing down disease progression and
359 increased survival (Beers et al., 2006; Boillée et al., 2006). In ALS the degenerating spinal
360 motor neuron bears its longest part, its axon, at the periphery in contact with macrophages and
361 other peripheral immune cells; while the soma is in the CNS being in contact with microglia.
362 This particularity of the spinal motor neuron led to the idea that if the peripheral immune system
363 could be modulated to act on disease progression, a very interesting and easier therapeutic
364 option will be discovered for all ALS cases. A recent article demonstrated that gene expression
365 patterns were very different across the disease in microglia and macrophages in SOD1^{G93A} mice
366 (Chiot et al., 2020) suggesting the two cell types play different roles in the disease. In this study,
367 replacement of peripheral SOD1^{G93A} macrophages by more trophic macrophages decreased
368 sciatic nerve macrophages activation and CNS microglial activation (Chiot et al., 2020). This
369 is particularly interesting as it shows that by modulating macrophages at the periphery it is
370 possible to act on CNS inflammation. Additionally, Chiot and colleagues demonstrated that
371 replacement of peripheral mutant macrophages by more trophic macrophages at the pre-
372 symptomatic stage delayed disease onset but was not sufficient to increase life expectancy,
373 whereas replacement done at disease onset increased survival of ALS mice (Chiot et al., 2020).
374 This temporality of replacement offers new insights into the time window that has to be targeted
375 to act on peripheral inflammation. Whereas most of the studies regarding the roles of
376 phagocytes were done with the mutant SOD1 mouse model, in which phagocytes showed
377 deleterious effects on disease progression, a recent study presented the neuroprotective effects
378 of reactive microglia in a TDP-43 mouse model (Spiller et al., 2018). All these results show
379 that the involvement of phagocytes is context-dependent and thus has to be studied in several
380 ALS models and in humans with different disease forms to understand their exact roles during
381 the disease course.

382 In FTD patients, two of the most commonly found mutated genes (GRN, C9orf72) have critical
383 roles in phagocytic and endocytosis machineries which are important for microglial proper
384 functions (Baker et al., 2006; Cruts et al., 2006; Shatunov et al., 2010; DeJesus-Hernandez et
385 al., 2011; Mok et al., 2012). Progranulin acts as an inflammatory modulator and its expression
386 is significantly up-regulated in microglia in several models of neuronal injury (Moisse et al.,
387 2009; Naphade et al., 2010; Tanaka et al., 2013). GRN knock out mice demonstrated aberrant
388 microglial activation upon stimulation and across aging (Yin et al., 2009; Martens et al., 2012;
389 Lui et al., 2016), leading to increased synaptic pruning or even neuronal loss. GRN deficient
390 mice also harbor obsessive-compulsive behaviors which seem to imply the TNF α signaling
391 pathway predominant in microglial cells in the CNS (Krabbe et al., 2017). This major immune
392 reaction can be alleviated through restoration of progranulin level. An overexpression of the
393 latter in a mouse model of sciatic nerve injury led to accelerated axonal regrowth, restoration
394 of neuro-muscular junctions, and recovery of sensory and motor functions (Altmann et al.,
395 2016). Regarding the second most common genetic form of FTD, studies of C9orf72 deficiency
396 in animal model showed a hyper activation of the innate immune system with increased
397 expression of IL-6 and IL-1 β in microglia and unregulated inflammatory genes in the spinal
398 cord (ORourke et al., 2016). Moreover, several studies reported a defective lysosomal system
399 with important accumulations in innate immune cells (Atanasio et al., 2016; Sullivan et al.,
400 2016; Moens et al., 2017; Atanasio et al., 2021). Together these results show a prominent role

401 of C9orf72 in microglial cells. The phagocytic machinery is essential for clearance of damaged
402 cell debris and maintenance of homeostasis. Disturbance in the proper functioning of this
403 machinery can lead to aberrant neuroinflammation and thus could directly contribute to the
404 disease process in FTD rather than being a secondary event to neuronal degeneration.
405 Nonetheless, these results need to be interpreted cautiously as both models do not match
406 haploinsufficiency found in FTD patients.

407 In humans, most studies focused on blood circulating monocytes as access to tissue resident
408 macrophages or microglial cells are impossible in alive patients.

409 In ALS most studies assessed monocytic populations at the periphery. An increase of classical
410 and a decrease of non-classical monocytes was observed not only in sALS and fALS patients,
411 in line with findings in the SOD1^{G93A} mice, but also in pre-symptomatic ALS mutant carriers
412 (Butovsky et al., 2012; Zondler et al., 2016), suggesting that this phenotype is an early event in
413 the pathology. Moreover, circulating monocytes of ALS patients presented functional defects
414 in phagocytosis and migration, and appeared to be skewed towards a more pro-inflammatory
415 phenotype with TNF α protein release positively correlating with progression rates, and IL-6
416 protein release positively correlating with disease burden (Zondler et al., 2016; Zhao et al.,
417 2017; Du et al., 2020). Deep RNA sequencing of ALS monocytes revealed unique
418 inflammation-related gene profiles, defects in migration and in the lysosomal pathway (Zondler
419 et al., 2016; Zhao et al., 2017). While aforementioned studies were carried out on blood
420 monocytes, a recent study observed that ALS monocytes-derived macrophages had increased
421 IL-6 and TNF α secretion levels when activated towards a pro-inflammatory state, suggesting
422 that monocytes-derived macrophages keep at least some of their *in vivo* characteristic defects
423 when in culture (Du et al., 2020). Other studies will be important now to better understand the
424 inflammatory states of ALS macrophages at different time-points of the pathology, as
425 monocytes are the easiest immune cells accessible from alive patients.

426 In FTD patients, a study revealed increased monocytes in the CSF of FTD patients in
427 comparison to controls with statistically non-significant increase in the proportion of non-
428 classical (CD14⁺, CD16⁺⁺) compared to classical monocytes (CD14⁺⁺, CD16⁻) monocytes
429 within the intrathecal compartment (Pawlowski et al., 2018). Elevated monocyte levels
430 correlated with structural cerebral defects in FTLD typical affected regions assessed by
431 structural MRI (Pawlowski et al., 2018). Further studies would be important to validate these
432 alterations at a larger scale.

433

434 ***Dendritic cells***

435 Dendritic cells (DC) orchestrate innate and adaptive complex organisations. DC are the most
436 efficient APC to activate T cells into specific lineages because they display more major
437 histocompatibility complex (MHC) classes and co-stimulatory molecules compared to others
438 APC (Inaba et al., 1997). In the periphery, immature DC seek for non-self antigens throughout
439 tissues. The encounter with a pathogen or a danger signal triggers their maturation, antigen
440 presentation on MHC II and migration to secondary lymphoid organs where they activate CD4
441 or CD8 T cells. On the contrary, at steady-state level, DC can present self-antigens to T cells to
442 avoid immune responses against self-proteins and induce tolerance. This process leads to the
443 induction of regulatory T cells (Treg) that express and secrete anti-inflammatory molecules to
444 reduce inflammation (Banchereau and Steinman, 1998).

445 In sALS and fALS post-mortem spinal cord' tissues, Henkel et al. reported the presence of
446 immature and mature DC as predominantly located in the degenerating cortico-spinal tracts,
447 with no staining in control tissues. In addition, they described an increased mRNAs levels
448 coding for DC surface markers and for the inflammatory chemokine CCL-2 (Henkel et al.,
449 2004). Interestingly, patients with a rapid disease progression had more DC transcripts
450 compared to patients with a slower progression. These data suggest an active recruitment of DC

451 by the inflamed CNS of ALS patients. A hypothesis is that besides the known CCL-2 production
452 (principally attributed to macrophage and microglia) and role of CCL-2 as a chemoattractant
453 for monocytes and T cells, DC could be an CCL-2 additional production source in ALS, and
454 therefore participate in immune cell recruitment on the inflammatory site. In agreement with
455 this hypothesis is that several studies described an increase of CCL-2 in ALS patient's CSF
456 (Baron et al., 2005; Kuhle et al., 2009; Tateishi et al., 2010; Gupta et al., 2011) and in the blood
457 of ALS patients a subpopulation of ALS patient-circulating DC showed an increased production
458 of CCL-2 in response to LPS (Rusconi et al., 2017).

459 Further analysis of circulating DCs in blood of ALS and FTD patients will be of great interest
460 if the different subpopulations of DC could be identified. Indeed, the DC family is composed
461 of several subpopulations with different phenotypes and functions. cDC1 have the ability to
462 highly cross-present antigen to CD8 T cells, while cDC2 are the most efficient DC subset to
463 polarize naive CD4 T cells (Haniffa et al., 2013). pDC are a main actor of antiviral responses
464 by the production of type 1 interferon (IFN-1) (Dzionek et al., 2000) and monocyte-derived DC
465 (MoDC) were shown to arise during inflammation. MoDC have been first observed in mice
466 (León et al., 2007) and then in humans under inflammatory physiological and
467 physiopathological conditions (Segura et al., 2013; Tang-Huau et al., 2018).

468 469 ***Mast cells***

470 Mast cells are long lived tissue resident cells, implicated in many different inflammatory
471 responses such as allergic reactions, parasite infection and physical injury. As they encounter
472 specific antigens, they activate and release numerous inflammatory mediators (i.e. histamine,
473 cytokines, lysosomal enzymes, ATP, serine proteases). Mast cells are also present in the CNS
474 where they have similar functions. They are generally located near structures mediating visceral
475 sensory or neuroendocrine functions or close to the blood-brain-barrier (BBB). In the spinal
476 cord under normal conditions, mast cells are present at the dura but not in the cord parenchyma
477 (Michaloudi et al., 2008). Mast cells are also first line effectors through which pathogens can
478 affect the gut-brain axis (Budzyński and Kłopotcka, 2014; Conte et al., 2020).

479 In the past years, mast cells received increased attention, as they appeared to be an early
480 responder of injury (Skaper et al., 2014). Moreover, as they are activated they are important
481 mediators of the microglial inflammatory response, astrocyte activation and potential neuronal
482 degeneration (Skaper et al., 2014; Zhang et al., 2016; Jones et al., 2019). Due to their vicinity
483 with the BBB they are also able to disrupt and permeabilise the BBB leading to toxin and
484 immune cells penetration exacerbating the inflammatory response.

485 In post mortem spinal cord tissues of ALS patients, increased Cox-2 mast cells - Cox-2 is a key
486 mediator of inflammatory reaction (Chen, 2010) - were detected while they were not present in
487 control subjects (Graves et al., 2004), suggesting that these cells play a role in the pathology.
488 In the SOD1^{G93A} rat model, accumulation of mast cells was observed in ventral roots and spinal
489 cords (Trias et al., 2018). Interestingly, mast cells were also found in periphery and in particular
490 in muscles, with infiltrations and degranulations correlating with paralysis progression (Trias
491 et al., 2017). Mast cells are thought to be recruited along the degenerating nerve by the Stem
492 Cell Factor (SCF) secreted from degenerating Schwann cells and reactive macrophages (Trias
493 et al., 2019). Very recently, another article showed that mast cells were interacting with
494 astrocytes and motor neurons expressing SCF in SOD1^{G93A} mouse model (Kovacs et al., 2021).
495 They also observed mast cells in the grey matter of ALS patient postmortem spinal cord tissues,
496 near to altered microvascular elements and surrounding motor neuron cell bodies (Kovacs et
497 al., 2021).

498 Pharmacological inhibition of CSF-1R and c-kit with Masitinib can reduce immune cell
499 infiltration and ameliorate NMJ integrity, showing an implication of these cells in the
500 axonopathy in periphery in the ALS pathology (Trias et al., 2016, 2020). Based on these

501 encouraging results, clinical trials were launched with Masitinib which is a tyrosine kinase
502 inhibitor targeting the SCF receptor (c-kit) and the platelet derived growth factor (PDGF)
503 (ongoing trials from AB Science).

504 These data suggest that mast cells participate to inflammatory reactions in the ALS pathology
505 both in the CNS and PNS. Whether they are a driver or an amplifier remains to be determined.
506 As at the symptomatic phase in the SOD1^{G93A} rats, mast cells have already increased in number,
507 their “early” implication in the pathology is suggested. It would be of great interest now to
508 assess their presence at the pre-symptomatic stage.

509 In FTD patients and models, not much is known about the implication of mast cells in the
510 pathology, but it wouldn't be aberrant to imagine mast cells increased reactivity as was observed
511 in Alzheimer's Disease patients (Harcha et al., 2021).

512

513 ***Natural killer cells***

514 Natural killer (NK) cells are classified as innate cytotoxic lymphocytes and are mainly known
515 for their ability to kill virus-infected cells and tumor cells (Abel et al., 2018). Few papers report
516 an increase of NK cells in the blood of ALS patients (Gustafson et al., 2017; Jin et al., 2020).
517 Very recently, Garofalo et al described an infiltration of NK-p46+ cells, a NK cell marker, in
518 the spinal cord and motor cortex of patients with sALS (Garofalo et al., 2020). They also
519 showed NK cell recruitment in the CNS of SOD1^{G93A} mice and CCL-2 was shown to be
520 involved directly in it or through other cell recruitment, since CCL-2 neutralisation led to a
521 decrease of NK cell infiltration. These data identified CCL-2 as an early damage signal of neural
522 tissue. The depletion of NK cells in SOD1^{G93A} mice, but also in TDP43^{A315T} mice, increased
523 survival and delayed paralysis onset. Functionally, control and SOD1^{G93A} NK cells had a
524 cytotoxic effect on SOD1^{G93A} motor neurons, but not on control motor neurons, suggesting a
525 kill-me signal coming from mutant motor neurons. Regarding the impact on other immune cells,
526 they showed that NK-depleted SOD1^{G93A} mice had a decreased number of microglia in spinal
527 cord ventral horns, with a less inflammatory profile. NK depletion also induced an increase of
528 regulatory T cells (see below) in the ventral horns (Garofalo et al., 2020). More studies deserve
529 now to be done to better understand the involvement of NK cells in ALS. In FTD patients, no
530 change of NK cell percentage in patient blood (Busse et al., 2017).

531

532 Altogether, these data support the critical role of innate immunity in ALS-associated
533 neurodegeneration. Phagocytes, mast cells, DC, and NK cells seem to participate and be
534 activated in early disease phases. The different therapeutic trials conducted to counteract
535 negative actions of these cells towards motor neurons in ALS patients are described below.

536

537

538 **Adaptive Immunity in ALS and FTD**

539

540 The adaptive immune system, unlike innate immunity, is highly specific to the particular
541 pathogen that induces it and can provide long lasting immune protection. This type of immunity
542 is strictly confined to vertebrates as it arises in evolution less than 500 million years ago (Alberts
543 et al., 2002). Amongst the cells that constitute this second line of immunity, major actors are T
544 and B cells that can be subdivided in many subclasses which can play different inflammatory
545 roles and serve different purposes during inflammatory events.

546

547 ***T lymphocytes***

548 The T lymphocyte population is composed of two main subpopulations: CD4 and CD8 T cells.
549 They are both characterised by the expression of CD3 and T-cell receptor (TCR) at their
550 membrane surface. During T cell activation, the TCR recognises antigen peptides presented by

551 APC on MHC II for CD4 T cells and MHC I for CD8 T cells. In addition to this complex signal
552 TCR/MHC/peptide, co-stimulatory molecules interactions (the main one being CD28 binding
553 to CD80/CD86) and cytokines are necessary for T cell activation (Kapsenberg, 2003).

554

555 CD4 T cells

556 CD4 T cells, also called T helper (Th) cells, help to set up an appropriate immune response
557 against the encounter pathogen. To this aim, they provide signals to other immune cells that
558 influence their activation and thus guide the immune response according to the pathogen to
559 evince. Naive Th cells are activated and polarised by APC, mainly DC, in secondary lymphoid
560 organs. They are classified in several subsets, the main ones being Th1, Th2, Th17 and Treg
561 cells. They are primarily defined by their effector cytokine productions and their transcription
562 factor pattern of expressions as described in Table 2.

563 In ALS, Th cells are described as major players in inflammation and disease progression. In the
564 1990s, CD4 T cell infiltrations were observed in spinal cords of ALS patients in proximity to
565 degenerating areas (Kawamata et al., 1992; Engelhardt et al., 1993). At this time, the different
566 Th cell subsets were not all discovered. More recent studies on patient's blood samples and on
567 some patient's CNS tissues focused more precisely on the different Th subpopulations.

568 Amongst the most deregulated subpopulations, Treg cells received a lot of attention. Treg cells
569 were found significantly reduced in ALS patient's blood compared to control subjects
570 (Mantovani et al., 2009; Henkel et al., 2013; Saresella et al., 2013; Sheean et al., 2018; Jin et
571 al., 2020). In their report describing significant decreases of FoxP3 mRNAs in circulating
572 leukocytes of ALS patients compared to healthy donors, Henkel et al. showed that the low
573 FoxP3 mRNA levels correlated with a rapid progression of the disease and a poor survival of
574 patients (Henkel et al., 2013). Interestingly, similar results were observed in the SOD1^{G93A}
575 mouse model with decreased Treg cell numbers and increased ones of other Th subsets along
576 disease progression (Beers et al., 2011; Zhao et al., 2012). Recently, Beers et al. went one step
577 further focusing on the impact of the disease on Treg functions (Beers et al., 2017). Treg are
578 known to suppress both innate and adaptive immune reactions detrimental to the host and in
579 particular they can suppress the activation/expansion of neurotoxic T lymphocytes. They
580 demonstrated that Treg isolated from blood of ALS patients were less effective in their capacity
581 to suppress proliferation of responder T lymphocytes. In addition, within the ALS patient group,
582 Treg cells of rapid progression patients (1-2 years) exhibited an even more reduced suppression
583 capacity compared to Treg cells of slow progression patients (4-6 years). Thus, Treg cell
584 suppressive capacity was inversely correlated with the disease rate (Beers et al., 2017). All these
585 results strongly suggesting a direct role of Treg in ALS led to the development of therapeutic
586 proposals to force Treg production (see below).

587 Th1 (or IFN- γ -producing CD4 T cells) and Th17 (or IL-17-producing CD4 T cells) cells are
588 increasingly considered as playing key roles in ALS inflammation. In most studies, these two
589 Th subsets were usually studied together because of their pro-inflammatory properties, despite
590 their distinct immunological functions. Interestingly, Th17 cells are known to be pathogenic in
591 many inflammatory diseases such as multiple sclerosis or inflammatory bowel disease (Wu and
592 Wan, 2020). In SOD1^{G93A} mice abnormal CD4 T cell activation and Th17 cell numbers were
593 described in the draining cervical lymph nodes prior to the onset of neurological symptoms (Ni
594 et al., 2016). In ALS patient's blood, both Th1 and Th17 cells were shown to increase (Saresella
595 et al., 2013; Jin et al., 2020) and IL-17 was measured in higher concentration compared to
596 controls (Rentzos et al., 2010). At the level of the CNS, Henkel et al. showed an increase of
597 Th1 mRNA markers in the spinal cord of ALS patients compared to controls. Interestingly, they
598 described an increase of T-bet mRNA in patients with rapid and slow progression of the disease
599 but an increase of the prototypic Th1 cytokine IFN- γ mRNA only in patients with rapid
600 progression of the disease (Henkel et al., 2013). Eventually, very recently, a functional study

601 focused on the effect of IL-17A, on WT or FUS-mutated motor neurons. The authors showed
602 that IL-17A decreased motor neurons survival and neurite network in a dose-dependent manner.
603 Treatment with anti-IL17A and anti-IL-17A receptor rescued neurotoxic effect of IL-17A
604 cytokine. Interestingly, all these observations were specific to IL-17A Th17 cytokine, IL-17F
605 treatment did not induce motor neuron degeneration (Jin et al., 2021).

606 Few studies reported a decrease of Th2 cells or Th2 associated markers in blood of ALS patients
607 (Henkel et al., 2013; Jin et al., 2020). In the same way as for Treg cells, Henkel et al. described
608 an inverse correlation of GATA-3 and IL-4 mRNA expressions with disease rate progression
609 (Henkel et al., 2013). On the contrary, Saresella et al. showed an increase of CD4 GATA3 cells
610 in patient's blood compared to healthy controls and no change in CD4 IL-4 cells (Saresella et
611 al., 2013). Moreover, another study described a positive correlation between the percentage IL-
612 13 cells within CD4 T cells and the disease progression rate and in the same line, an inverse
613 correlation between the percentage IL-13 cells within the CD4 T cell population and the
614 ALSFRS-R score (Shi et al., 2007). These results are of particular interest as the two major Th2
615 cytokines, IL-4 and IL-13, have been shown to increase CCL-2 production by activated primary
616 rat microglia and by an activated human monocytic cell line THP-1 (Szczepanik et al., 2001).
617 Whether Th2 cells or Th2 cytokines have a neuroprotective or a neuro-inflammatory role seems
618 to depend on the context.

619 Taken together, these data show that the composition of the total T cell population is modified
620 in ALS patients compared to controls. The balance between the different subsets of Th appears
621 to evolve over disease progression. Interestingly, specific correlations seem to exist between
622 expressions of specific mRNA Th subtypes and ALS patient's disease progression rate (Henkel
623 et al., 2013). These markers could be of great interest as new biomarkers to monitor disease
624 progression rate in ALS patients.

625 In FTD patients, one study described a decrease of total T cell (CD4 and CD8) percentages in
626 blood samples (Busse et al., 2017). Another study described a specific decrease of cytotoxic T
627 lymphocyte antigen-4 (CTLA-4) CD4 T cells and no change of CD28 CD4 T cell frequency in
628 patients' blood (Santos et al., 2014). CD28 is a co-stimulatory immune checkpoint that
629 promotes T cell activation, proliferation and response, whereas CTLA-4 is an immune
630 checkpoint molecule exerting an inhibitory function on T cell proliferation and function.
631 Notably, these two molecules compete for the same receptor, namely CD80/CD86 (Chambers
632 et al., 2001). Hence, this CTLA-4 deficiency could suggest a possible exacerbated activation of
633 these CD4 T cells in FTD patients. Despite these studies, little is known about CD4 T cells in
634 FTD. Given their rising importance in ALS, it would be worth investigating Th subsets in more
635 details.

636 637 CD8 T cells

638 The main function of CD8 T cells, also called cytotoxic T cells (CTL), consists in the
639 elimination of cells infected with intracellular pathogens like viruses but also in elimination of
640 tumor cells. CD8 T cells have been shown to play a determinant role in neurodegenerative
641 diseases like multiple sclerosis (Huseby et al., 2012), but studies about their involvement in
642 ALS remains sparse.

643 Similarly to Th cells, CD8 T cell infiltration were observed in the spinal cord and in the brain
644 of ALS patients (Kawamata et al., 1992; Engelhardt et al., 1993; Fiala et al., 2010).
645 Contradictory results exist on the percentages of CD8 T cells in ALS patient's blood. Some
646 studies showed an increase (Rentzos et al., 2012; Jin et al., 2020) while others suggested a
647 decrease (Mantovani et al., 2009) or no change (Murdock et al., 2017). In SOD1^{G93A} mice, CD8
648 T cells were shown to progressively infiltrate spinal cords (Chiu et al., 2008; Beers et al., 2011;
649 Figueroa-Romero et al., 2019), while Beers et al. observed CD8 T cell infiltration only at
650 disease end-stage in SOD1^{G93A}/CD4^{-/-} mice (Beers et al., 2008). In 2018, Nardo et al. revealed

651 a role of CD8 T cells and MHC I (expressed on APC and necessary for CD8 T cell activation)
652 in disease progression. In SOD1^{G93A} mice defective for CD8 T cells and expressing little MHC
653 I, a dual role of MHC I was shown with specific pathogenesis in the CNS and the PNS. In the
654 CNS, without CD8 T cell infiltration and MHCI microglia, inflammation was reduced, paralysis
655 of forelimbs was delayed and survival of mice was extended. On the contrary in the PNS motor
656 axon stability was affected leading to the acceleration of muscle atrophy (Nardo et al., 2018).
657 This suggested that MHC I-dependent interaction between either CD8 T cells or microglia is a
658 key factor triggering neuroinflammation, and also that a slow-down of disease progression may
659 be obtained through activation of the MHCI signaling in the periphery to protect the axon-
660 muscle connectivity.

661 In FTD, parenchymal CD8 T cells were detected in the cortex of patients with tau^{P301L} mutation
662 (Laurent et al., 2017). Same observations were made in a mouse model of tauopathy and
663 interestingly the depletion of peripheral T cells using an anti-CD3 antibody abolished CD8
664 infiltration in the cortex but also restored cognitive capacity of mutant mice, suggesting a
665 crucial role of these T cells in cognitive impairment (Laurent et al., 2017).

666

667 ***B lymphocytes***

668 B cells are the center of adaptive humoral immunity and mediated extracellular pathogen
669 elimination by antigen-specific immunoglobulin production. Evidences of B cell implication in
670 ALS is very limited. Auto-antibodies against proteins of spinal cord cells were detected in the
671 serum and the CSF of some ALS patients (Niebroj-Dobosz et al., 2004, 2006; Puentes et al.,
672 2014). Auto-antibodies have also been detected in SOD1^{G93A} mice without any impact on
673 disease progression (Naor et al., 2009). It remains unclear if auto-antibodies may be a
674 consequence of motor neuron death and if they could participate to motor neuron degeneration
675 and inflammation aggravation. A recent study investigated the impact of regulatory B (Breg)
676 cell adoptive transfer on disease progression in SOD1^{G93A} mice (Pennati et al., 2018). As Treg
677 for CD4 T cells, Breg are immunosuppressive B cells involved in immune homeostasis and
678 tolerance maintenance (Rosser and Mauri, 2015; Peng et al., 2018). Breg adoptive transfer
679 increased Treg cell percentage in the CNS at five months after the transfer, but no impact on
680 the survival was reported (Pennati et al., 2018). It would be of particular interest to understand
681 why and how Breg cells impact Treg cells. From what was discussed above, Treg cells are
682 decreasing overtime in ALS patients. Thus, being able to increase the number of Treg cells,
683 even in an indirect manner, could be of interest to delay disease progression in patients. In FTD
684 patients, only one study reported a decrease of B cell percentage in the blood of patients (Busse
685 et al., 2017). More investigations are now necessary to understand their role in FTD. Indeed,
686 and more globally, current studies about immunity in FTD were more focused on dementia and
687 FTD phenotypes were mainly compared to AD phenotypes. Considering the common spectrum
688 of ALS and FTD, more comparative studies would help to understand whether these two
689 neurodegenerative diseases share some immune deregulation and how inflammation really
690 affects disease progression in FTD.

691

692 In conclusion, what is better understood today is whom and where immune cells could play a
693 role during disease progression (Figure 1, Table 3) and what they could secrete to protect
694 neurons or to eliminate debris and dead cells (Table 1). Whereas Table 3 summarises
695 dysfunctions of the different immune cell types in ALS and FTD pathologies, Table 1 lists
696 known dysregulated molecules. However, which cell is responsible of a specific secretion as
697 well as which cells respond to specific factors remains largely elusive. Direct interactions
698 between neurons and immune cells are still not well understood, as it remains impossible to
699 have access to human brain and spinal cord tissues in alive patients. Yet, as ALS is an incurable
700 disease with a very short life expectancy, clinical trials were launched and since the late 90's

701 several immune modulatory drugs have been tested in clinical trials for ALS even if the mode
702 of action of molecules were not fully understood. In the next parts, we will present the main
703 clinical trials and discuss their limits and their future in particular with regard to what we expect
704 to learn from future studies with iPSC-derived modeling approaches.

705
706

707 **CLINICAL TRIALS TO MODULATE THE IMMUNE SYSTEM**

708

709 While inflammation appears as a major component of ALS and FTD pathologies, which could
710 be targeted in order to delay disease progression, the complexity of the whole immune system
711 and the numerous cell types involved in the different inflammatory pathways make difficult the
712 understanding of the real impacts of therapeutic drugs on disease phenotypes. This being, with
713 the urgent need to find a cure or even a treatment to delay disease progression, different trials
714 with two types of strategies have been deployed to act on inflammatory components. The first
715 strategy implies immune modulatory drugs and the second strategy implies transplantation of
716 different cell types into patients. All clinical trials can be found on <https://clinicaltrials.gov>
717 (below, each reference “NCT1234...” mentions one specific trial).

718

719 **Immune Modulatory Drugs**

720

721 As shown in Table 4 and Figure 2, a large majority of considered immune modulatory drugs in
722 ALS clinical trials are repositioned molecules previously tested in other diseases harboring
723 inflammation (Huntington’s disease, Multiple Sclerosis, rheumatoid arthritis). Their
724 mechanisms of action are not presented here as they were described previously in details
725 (Khalid et al., 2017). Among tested molecules, most are antagonists of pro-inflammatory
726 pathways reported to be deregulated in ALS patients. While some tested molecules induced
727 adverse effects, most of them demonstrated safety and tolerability but their efficacy remains
728 limited or negative. To date most of the proposed molecules have a very large panel of targets
729 and act on signaling pathways shared by many cells. While the two molecules accepted on the
730 market (Riluzole and Edaravone) claim to act on neuroprotective functions for motor neurons;
731 it turns out that for immune modulating trials the targets are broad and can have consequences
732 on a large number of functions and thus possible uncontrolled impact on the disease (Table 4).
733 Today, eleven trials are either recruiting and/or active, targeting one or several molecules and
734 pathways. Among these trials, one ongoing phase II is with low IL-2 dose (ld-IL-2) targeting T
735 cells. This trial has already shown some encouraging results demonstrating increased Treg cells
736 (Camu et al., 2020). In another phase II trial, RNS60, an anti-inflammatory drug, targets the p-
737 Akt pro-survival pathway and NFkB (Paganoni et al., 2019). Compared to the ld-IL-2 trial the
738 latter has 6 defined cell targets and 3 possible others, a non-specificity that could be responsible
739 for uncontrolled modulations of pathways. Fortunately, adverse effects were not reported. A
740 Phase II/III trial (Ibudilast) uses a small molecule already tested in patients with Multiple
741 Sclerosis. Contradictory results were reported and further studies are now necessary to
742 understand the exact impact of Ibudilast on patients (Chen et al., 2020; Babu et al., 2021).
743 Finally, a phase III trial (Ravulizumab) uses a monoclonal antibody targeting the C5
744 complement to decrease inflammation and results are eagerly awaited.

745 There are several reasons that may explain the limited success of each trial and current
746 limitations have to be taken into account in the ongoing and future trials. First, most of the
747 clinical trials include patients with very heterogeneous disease states (in terms of disease form
748 (sporadic, familial), durations, and site of onset). This is one reason why most studies do not
749 include ALS-FTD patients. Mechanisms involved in the pathological progression at different
750 disease steps may vary between patients and thus modulating inflammation with such

751 heterogeneity may erase effects attributable to one subgroup. Edaravone is a good example. No
752 beneficial effect was reported from the initial clinical trial conducted in a heterogeneous ALS
753 population; however, post-hoc analysis demonstrated beneficial effects in a clinical subset of
754 ALS patients (Okada et al., 2018; Abraham et al., 2019). Second, the inflammatory reaction in
755 patients is suspected to switch from protective to deleterious during disease progression. This
756 parameter remains very difficult to monitor as there are no validated biomarker able to assess
757 the disease state. Fortunately, recent advances in imaging techniques bring new hope for future
758 trials. For instance, a recruiting phase II clinical trial will evaluate the brain microglia response
759 in ALS patients by TSPO binding following multiple doses of BLZ945, an antagonist of the
760 CSF1 receptor, using PET with [11C]-PBR28 ([REDACTED] Other markers closely
761 monitored and already measured in patients are concentrations of neurofilaments light chain
762 (NF-L) and heavy phosphorylated chains (pNF-H) in blood and CSF (Gaiani et al., 2017;
763 Benatar et al., 2018; Huang et al., 2020). A consensus on pNFH as an early biomarker should
764 give the possibility to better follow the effects of any drug on specific markers (Benatar et al.,
765 2020). The future relies on the urgent need to identify specific biomarkers linked to different
766 ALS forms and to be able to identify when a molecule or another can have a positive impact on
767 the disease progression in each patient. In the past clinical trials, some positive effects of drugs
768 might have been missed due to the lack of these biomarkers. Finally, as 90% of ALS cases are
769 sporadic, the diagnosis is done when symptoms are already present and when inflammatory
770 events are already well advanced. In this situation, having the possibility to follow markers in
771 known mutated patients before any symptom appearance could bring very important insights.
772 Chipika et al. (Chipika et al., 2020) have performed a systematic review of existing
773 presymptomatic studies. They showed that studies suffered from small sample size, lack of
774 disease controls, and rarely cohorts were followed until symptom manifestations. Despite these
775 limitations, objectives of these kind of studies are to be able to propose earlier diagnosis and
776 thereby earlier therapeutic interventions or enrollment in clinical trials with homogenous sub-
777 groups of patients. Personalised therapies are currently emerging, as exemplified by the antisense
778 oligonucleotide Tofersen for SOD1 ALS patients (Miller et al., 2020) and even for
779 presymptomatic SOD1 carriers in a new ongoing trial (NCT04856982).

780

781 **Cell Transplantation**

782

783 This second therapeutic approach implies transplantation of specific cell types in patients. More
784 than 30 trials are listed on <https://clinicaltrials.gov>. Morata-Tariffa et al. (Morata-Tariffa et al.,
785 2021) provide in 2021 a meta-analysis of stem cell therapy in ALS. Among cell types available
786 for transplantation, mesenchymal stem cells (MSC) have been the most widely tested
787 autologous transplants through different techniques and route of administration (i.e. spinal cord,
788 frontal lobe, intrathecal) (Mazzini et al., 2008; Deda et al., 2009; Kim et al., 2014). MSC
789 represent interesting cell candidates as they are easy to culture *in vitro* and are able to be
790 differentiated into bone stroma, cartilage, ligament and fat depending on the surrounding
791 molecules (Deans and Moseley, 2000; Verboket et al., 2018). Moreover, the latter express
792 cytokines, growth factors that could be involved in therapeutic contributions for neuronal
793 protection and act on local inflammation (Djouad et al., 2007; Suzuki et al., 2008). Besides
794 MSC, bone marrow cells (BMC) were also transplanted in patients (Deda et al., 2009; Ruiz-
795 López and Blanquer, 2016). These cells are a mixture of several cell types, containing
796 lymphocytes, monocytes, and progenitor cells, giving them a strong regenerative potential
797 (Verboket et al., 2018). Preclinical studies in animal models have shown safety and tolerability
798 after transplantation of both cell types, as well as beneficial effects on pathogenic signatures
799 (Kim et al., 2010; Lewis and Suzuki, 2014). In ALS trials, conclusions of the therapies have
800 been difficult to draw, especially because of the small number of patients included (Mazzini et

801 al., 2008; Deda et al., 2009; Karussis et al., 2010). Analysis of Morata et al. showed that the
802 best results were obtained with intrathecal injections of MSC in patients but with only transient
803 positive effects on clinical progression (ALSFRS-scores). One major drawback of these
804 transplantations is that after all interventions respiratory functions were negatively impacted.
805 As described in the previous section, a clinical trial administrating IL-2 was launched in order
806 to force the production of Tregs in ALS patients. No adverse effects were reported and an
807 increased Tregs number was observed (Camu et al., 2020). Transposition to cell therapy has
808 then been considered. Thanks to set up protocols allowing Tregs *ex vivo* expansion with anti-
809 CD3/CD28 beads, rapamycin and IL-2 (Alsuliman et al., 2016) a phase I clinical trial was
810 launched to test further transplantation of *ex vivo* expanded Tregs. First results suggested safety
811 with no clear conclusion as the study was underpowered for efficacy and did not have placebo
812 or controls. However, from the results observed in the 3 transplanted patients, Tregs
813 transplantation induced an increase in Tregs percentages, amelioration of Tregs suppressive
814 functions and a slowing down in the decrease of the ALSFRS-score. Unfortunately, these
815 effects disappeared between rounds of Treg cell injections (Thonhoff et al., 2018). Nonetheless,
816 a phase II is currently undergoing including 12 patients with placebo controls, despite
817 cumbersome protocols for patients.
818 The use of such autologous cell transplantation therapies could be very interesting as they
819 appear more adapted to a large number of ALS patients and do not necessitate a direct access
820 to the CNS. Some trials, notably with MSCs (NCT03280056), currently on their way may offer
821 in the near future new possibilities for ALS patients. However, for all cell types proposed for
822 cell therapies, it seems imperative today to come back to preclinical studies to optimise cell
823 types, administration routes and cell distributions, and to better understand their mechanisms
824 of action.

825
826

827 **iPSC TO MODEL IMMUNE REACTIVITY IN ALS AND FTD**

828

829 As described in the above paragraphs, immune reactions implying several cell types arise during
830 disease progression in patients with ALS and FTD. Post-mortem tissues showed activations and
831 infiltrations of specific cell types, while CSF and blood samples analysis assessed of the
832 production and secretion of many molecules involved in inflammatory responses. As the latter
833 can be beneficial or deleterious depending on signals in their environment, performing
834 longitudinal studies would be very informative. Unfortunately, the particularity of ALS and
835 FTD diseases, compared to other neurodegenerative diseases, is that disease duration is very
836 short. The consequence is that it is nearly impossible to obtain more than one blood or CSF
837 sample per patient, making longitudinal studies almost impossible. To go one-step further and
838 decipher specific mechanisms that could be new targets of clinical trials, direct or indirect
839 interactions between affected neurons and the different immune cells have to be determined.
840 However, whereas blood sampling may allow access to some immune cells of patients, biopsies
841 of the brain and the spinal cord regions in which neurons are affected and in which tissue
842 resident or infiltrated cells have to be analysed, are not possible. Thanks to the iPSC technology
843 described 14 years ago, researchers can now have access to human pluripotent cells that can be
844 differentiated in theory into any cell type of the body. “In theory” as we know now that each
845 protocol to differentiate iPSC into specific cell subtypes necessitates years of technical
846 developments. Nevertheless, iPSC offer the unique opportunity to study intrinsic defects in iPSC-
847 derived mutant cells and interactions between different cell types in 2D and 3D co-cultures.

848

849 **iPS-derived neurons**

850

851 Until now, the vast majority of studies focused on iPS-derived neurons. Many reviews already
852 present the unique capabilities of iPS-derived neurons to capture some key features of ALS and
853 FTD in motor neurons and cortical neurons, respectively (Bohl et al., 2016; Lee and Huang,
854 2017; Hawrot et al., 2020; Lines et al., 2020). When protocols to generate iPS-derived neurons
855 first appeared they allowed production of generic neurons with very poor purity. As technology
856 advances many subtypes of neurons are now able to be generated; thus allowing to better
857 understand the dichotomy between, the ubiquitous expression of mutant genes in patients' cells
858 and the death of specific subtypes of neurons (Sances et al., 2016). Nevertheless, there are still
859 improvements in progress to obtain in culture the most specific neuron subtypes. For example,
860 for motor neurons, it is today possible to characterise in cultures if generated motor neurons are
861 those of the lateral or medial motor column (LMC or MMC) (Amoroso et al., 2013). In fact
862 Mouilleau et al. deciphered mechanisms allowing the expression of HOX genes involved in the
863 identities of motor neurons along the rostro-caudal axis of the spinal cord (Mouilleau et al.,
864 2021). These different findings open the way to generate specific cervical to brachial motor
865 neurons of the LMC or MMC that may be differentially affected in ALS patients. Interactions
866 between neurons and astrocytes were also reported in some publications (Lee and Huang, 2017;
867 Lines et al., 2020) and different results were reported regarding toxicity of astrocytes towards
868 motor neurons depending on the studied ALS form, showing the necessity to pursue
869 investigations.

870

871 **Innate immune cells**

872

873 Innate immune cells, including macrophages, dendritic cells, mast cells and NK cells, were the
874 first immune cells generated from human stem cells (iPSC and embryonic stem cells (ESC)).

875

876 ***Macrophages and microglial cells***

877 As for neurons, protocols to generate macrophages have been progressively improved thanks
878 to concomitant advances in the knowledge of macrophage biology, origin and diversity. For
879 ALS, two subtypes of macrophages are of interest: microglial cells, the macrophages of the
880 CNS, and peripheral macrophages located along the motor axon from the ventral root to the
881 neuromuscular junction. For FTD, only microglial cells are of interest at first sight. To obtain
882 macrophages or microglia from patients, first protocols were based on human monocytes
883 isolated from blood samples and activated with cytokines *in vitro*. However, it is now known
884 that these macrophage-like cells are not capable to model tissue-resident macrophage
885 populations that arise from embryonic precursors (Lavin et al., 2014; Hagemeyer et al., 2016;
886 Lee et al., 2018). Moreover, macrophage activation exists as a spectrum of phenotypes and
887 functional states challenging to reproduce in culture (Ginhoux et al., 2016). As an alternative
888 option and as an infinite source of patient-specific cells, ESC and iPSC were tested for their
889 capacity to be differentiated into macrophage-like cells. A great difficulty for such iPSC
890 protocol developments was the capacity to generate tissue-resident macrophages and in
891 particular macrophages of the CNS. Indeed, microglia and peripheral macrophages have
892 different developmental origins and are in very different environments *in vivo*, suggesting that
893 different protocols should be set up to mimic the diverse macrophage subtypes. For microglia,
894 an additional problem relies in the impossibility to compare iPS-derived cells to a “gold
895 standard” human microglial cell. Indeed several factors render it complicated: first of all, it is
896 almost impossible to obtain healthy tissue from an adult person and therefore to study the
897 microglial cells in a non-pathogenic environment; second, the amount of cells collected is
898 generally low, especially in non-inflammatory tissue; lastly, it has been shown that the
899 microglial cells gradually lose their cell identity in culture when they were taken out of their
900 microenvironment. Many reports and reviews describe and compare published protocols

901 allowing the generation of functional macrophage-like cells (Haenseler and Rajendran, 2019;
902 Hasselmann and Blurton-Jones, 2020; Hedegaard et al., 2020; Lyadova et al., 2021). Clearly,
903 iPSC require specific environmental signals for proper differentiation and maintenance of their
904 identity (Bohlen et al., 2017; Gosselin et al., 2017), suggesting that for ALS and FDT studies,
905 iPSC-derived microglia have to be co-cultured with either motor neurons or cortical neurons,
906 respectively.
907 iPSC-derived macrophage/microglia monocultures phenotypic defects were observed in different
908 models (Haenseler and Rajendran, 2019). Zhao et al. studied iPSC-derived macrophages from
909 C9ORF72^{(G4C2)ⁿ} patients and control subjects and compared their immune-suppressive
910 functions (Zhao et al., 2020). Their results demonstrated that immunosuppressive functions of
911 ALS macrophages were similar to controls, suggesting that the mutation did not influence this
912 function in macrophages. Some other reports described (i) 2D co-cultures of microglia with
913 neurons to mimic their neural environment, (ii) microglia generation in brain or spinal cord
914 organoids, or (iii) xenotransplantation of microglial-like cells (Hasselmann and Blurton-Jones,
915 2020; Fattorelli et al., 2021; Tan et al., 2021). Reports in 2D cultures between neurons and iPSC-
916 derived microglia studied for examples their morphology and migration, their inflammatory
917 responses and clearance capacity. Similarly, in 3D cultures and in organoids, observations were
918 about migration, activation, proliferation, and physical interactions of microglia with other cells
919 (Haenseler and Rajendran, 2019). Most of current studies focused on microglia addition and
920 modeling of Alzheimer's or Parkinson's defects, making the proof that the different iPSC based
921 models could bring new knowledge for ALS and FTD. Recently, the generation of microglia-
922 like cells in human sensorimotor organoids derived from ALS iPSC was reported (Pereira et
923 al., 2021), proving that these cells can arise and be phenotypically studied in these models.
924

925 ***Dendritic cells, mast cells and NK cells***

926 As described above, the DC family is composed of several subpopulations with different
927 phenotypes and functions. To study DCs, it is possible, like for macrophages, to generate
928 primary DC from peripheral blood-derived monocytes, and then to mature them into distinct
929 DC subsets *in vitro* (Romani et al., 1994; Sallusto and Lanzavecchia, 1994). However, for ALS
930 and FTD patients, the same limitation as of macrophages remains (few blood samples per
931 patient), which can be overcome with iPSC. In their review of 2020, Ackermann et al. describe
932 the different protocols to generate DC and their subpopulations from iPSC (Ackermann et al.,
933 2020). DCs were shown to be morphologically and functionally similar to their *in vivo*
934 counterparts. This model will be very helpful considering the limited availability of DC in blood
935 and tissues. Interestingly, the generation of DC offers the possibility to investigate their
936 interactions with T cells derived from the same patient, as iPSC-derived DC were shown to be
937 able to stimulate allogeneic naïve T cells and autologous antigen-specific CD8⁺ T cells.

938 Few protocols exist for human iPSC differentiation into mast cells (Kovarova et al., 2010;
939 Igarashi et al., 2018; Kauts et al., 2018; Ikuno et al., 2019). First protocols were long, with low
940 yield, and a production of immature cells. The most recent protocol (Bian et al., 2021) uses a
941 sequential co-culture system which allow short-term cultures and efficient production of
942 scalable quantities of functional mature mast cells. The latter were identified as a specific
943 subtype of mast cells. Indeed, 3 types of mast cells have been classified in humans according
944 to their protease content and location. The success of the Bian's protocol relies in the
945 identification of a new pathway of progenitor generation allowing the production of mature
946 mast cells exhibiting the strongest innate immune responses. Their working hypothesis was
947 based on developmental studies that found that mast cells had different origins in mouse
948 models, suggesting the same difference in humans (Gentek et al., 2018; Li et al., 2018). Thus,
949 these results proposed two waves of human mast cells development, reminiscent of the different
950 macrophages' generation waves. Alike macrophages, mast cells mature in tissues under the

951 influence of the local environment, suggesting that co-cultures with neurons might be the more
952 relevant study model for ALS and FTD.

953 A recent review summarises protocols to obtain NK cells from human iPSC (Shankar et al.,
954 2020). iPSC-derived NK cells were shown to be phenotypically similar to primary NK cells as
955 well as regarding their effector function. CD56 is a marker often used to define human NK cells
956 (murine NK cells are CD56 negative). Two subpopulations (dim and bright) have been
957 identified and play different roles (CD56 bright cells exhibit high cytokine secretion while
958 CD56 dim exhibit high cytotoxicity). As it is unclear if these both cell types are generated from
959 iPSC, further investigation will be necessary, before using those cells to conduct disease
960 modeling.

961

962 **Adaptive immune cells**

963

964 Whereas protocols to generate cells of the innate immune system were set up quite rapidly, the
965 differentiation of iPSC into cells of the adaptive immune system (T and B lymphocytes) is much
966 more challenging (Montel-Hagen and Crooks, 2019). Those cells originate from hematopoietic
967 stem cells that are able to give rise to myeloid and lymphoid progenitors. Regarding B
968 lymphocytes, first protocols just emerge and allow only to model the earliest stages of B lineage
969 development (French et al., 2015; Böiers et al., 2018; Richardson et al., 2021). For T
970 lymphocytes, whereas the first stages of iPSC differentiation into T progenitors were rapidly
971 set up, some cell specificities are difficult to reproduce to obtain *in vitro* mature T lymphocytes.
972 The particularity of the T-cell lineage is that the first stages of their differentiation occurs in the
973 thymus. To reproduce the thymus environment and differentiate iPSC into T progenitors, co-
974 cultures were performed between iPSC and stromal cells that express a Notch ligand. Notch
975 was shown to be essential to induce T cell differentiation of progenitors, at the expense of B-
976 cell differentiation. Recently, a stromal-cell free protocol was developed to generate the T cell
977 lineage from blood-derived CD34-positive cells (Nianias and Themeli, 2019; Netsrithong et al.,
978 2020), a strategy that has now to be transposed in iPSC differentiation protocols. Additionally,
979 lymphocytes express at their surface different receptors forming a repertoire (TCR for T cell
980 receptors). The latter is crucial for the response of T lymphocytes in an antigen-specific manner
981 to an unlimited number of unknown pathogens encountered throughout life. Interestingly iPSC-
982 derived T progenitors were shown to express a broad TCR repertoire (Chang et al., 2014),
983 suggesting that each iPSC clone have its own TCR repertoire. Current limitations of the iPSC
984 protocols rely in the maturation of T lymphocytes and their positive selection. This selection
985 consists in the maturation of double positive CD4-CD8 lymphocytes into simple positive cells.
986 Whereas iPSC protocols not clearly demonstrated their ability to mimic this positive selection,
987 a recent improvement was proposed with the generation of 3D artificial thymic organoids which
988 allowed the generation and maturation of conventional T cells (Montel-Hagen et al., 2019).
989 Having in mind the different roles played by T cell subtypes in neurodegenerative diseases, it
990 will be crucial now to generate IPS-derived Th1, Th2, Th17 or Treg cells. Once generated,
991 those cells will be of great interest for ALS and FDT modeling.

992

993

994 **CONCLUSIONS AND PERSPECTIVES**

995

996 ALS and FTD are two neurodegenerative diseases belonging to a common disease spectrum.
997 There is no treatment for these fatal diseases and life expectancy of patients is shorter compared
998 to the one of patients affected by other neurodegenerative diseases. The main conclusion from
999 decades of studies on patients, animal models and iPSC, is that ALS or FDT are heterogeneous
1000 at several levels and have to be seen as a group of diseases rather than a single one, at least

1001 regarding mechanisms leading to neuron degeneration. To date ALS or FTD patients are often
1002 still classified based on external clinical signs and this is mainly due to a lack in early
1003 biomarkers that could help to better classify patients. What we hope having shown in this review
1004 focused on ALS and FTD is the high complexity of inflammatory events intermingled one to
1005 another, a complexity that may be extended more largely to all neurodegenerative diseases.
1006 Many studies have identified inflammatory markers in patients, animal models and iPSC,
1007 nonetheless we still don't really understand if the inflammation is a driver or consequence of
1008 the pathology. Although some key aspects start to be lighted, questions remain: when exactly
1009 the inflammation is beneficial or detrimental? Which immune cells are responsible for the
1010 inflammatory responses? How the inflammatory events may differ between diverse genetic
1011 forms, especially in patients harboring mutations in genes known to be linked with immune
1012 functions (C9orf72, TBK1, GRN ...)?

1013
1014 The observation today is that decades of clinical trials modulating immune functions have failed
1015 to prove their beneficial effects to patients. Some explanations linked to pre-clinical protocols
1016 may explain these failures, such as the lack of power in numerous studies and the use of one
1017 single animal model that might not recapitulate all disease forms. Also, the temporality of drug
1018 administration is often in early or pre-symptomatic stages in pre-clinical studies, a stage that
1019 cannot be targeted in enrolled patients. In this review we wanted also to focus the tested immune
1020 modulatory drugs together with their unstudied potential targets that could have impact on the
1021 disease. Indeed, most of the tested molecules target generic receptors and consequently share
1022 mechanisms of action with several cell types, making difficult the monitoring of the real effects
1023 of the therapeutics. Our conclusion is that we still lack diagnostic tools and knowledge
1024 regarding the inflammatory events in ALS and FTD to efficiently modulate these events. The
1025 multicellular implication is now widely accepted knowing that most genes are ubiquitously
1026 expressed. Thus, the use of a single drug targeting one pathway may be outdated. More
1027 personalised medicine begins to appear as a mean of integrating all factors of heterogeneity.

1028
1029 If ALS and FTD animal models allow the study of genetic forms of the diseases, a lack in
1030 animal models recapitulating sporadic cases, remains. Additionally, difficulties in accessing
1031 CNS cells and the rapidity of disease progression make difficult to perform studies with
1032 patients. iPSC are now offering unique opportunities to bypass these hazards. With the
1033 development of iPSC differentiation protocols to generate immune cells, researchers have now
1034 at their disposal tools to answer questions about interactions between ALS- or FTD-affected
1035 neurons and immune cells in a human context and to study the temporality of these events. Of
1036 course, these protocols are still very new and imperfect: cultures are not always pure, some cell
1037 subtypes can still not be generated and iPSCs are known to have a more embryonic than adult
1038 genome memory (as opposed to transdifferentiated cells that do not go through an embryonic
1039 stage for their generation). This being, the current literature since several years has made the
1040 proof that it is possible to model diseases like ALS and FTD with iPSC-derived neurons. In the
1041 recent years, several drugs have been tested and approved for clinical trials based on preclinical
1042 studies on iPSC-derived neuronal cells (Fujimori et al., 2018; Okano et al., 2020). With regard to
1043 the innate and adaptive immune cells derived from iPSC, first papers showed that the generated
1044 cells were functional, more or less mature, and some functional intrinsic defects were shown,
1045 validating the use of these cells to identify further unknown mechanisms. To go one-step
1046 further, co-cultures are necessary to study interactions with neurons. Since few years, co-
1047 cultures, 3D cultures (e.g. microfluidic chips) as well as 3D brain and spinal organoids (Hor et
1048 al., 2018; Tan et al., 2021) are developed and available to study interactions between either
1049 motor neurons or cortical neurons affected mainly in ALS and FTD respectively, and the
1050 various immune cells. Even if iPSC-derived macrophages/microglia were the first generated

1051 immune cells, there is no report yet about iPSC modeling of ALS or FTD macrophage/microglia
1052 co-cultures with neurons. In their recent study, Pereira et al. describes the generation of human
1053 sensorimotor organoids with iPSC from ALS patients. They showed that these organoids
1054 contain microglia-like cells, but their study was not focused on the phenotypes of these cells,
1055 but rather on defects at the level of neuromuscular junctions (Pereira et al., 2021). Another
1056 study by Bowles et al. (Bowles et al., 2021) describes the generation of cerebral organoids from
1057 iPSC of 3 FTD patients mutated in the tau gene. Signs of neurodegeneration, fewer excitatory
1058 neurons and elevated levels of inflammation were observed in 6-month old cerebral organoids,
1059 showing that it was possible to recreate much of the damage seen in FTD. Moreover, they were
1060 able to prevent neuron death with an experimental drug. Even if microglial cells were not
1061 studied directly in these organoids, an inflammatory response was observed suggesting that this
1062 pathological feature could be modeled in such 3D models. In the other hand, interestingly, for
1063 diseases appearing late in life, Chen et al. (Chen et al., 2021) recently showed that exposure of
1064 brain models to serum mimics age associated blood-brain barrier breakdown, providing a
1065 platform to look for effective treatments for disorders with an age component like ALS and
1066 FTD.

1067 As a final word, the iPSC technology grants new prospects to better understand and model
1068 inflammatory events in FTD and different ALS forms. 3D cultures are offering a new era of
1069 researches with more integrated models, which we hope will lead to new ways to do drug
1070 screening and soon offer adapted therapeutic opportunities for ALS and FTD patients.

1071

1072

1073 **AUTHOR CONTRIBUTIONS**

1074 All authors listed have made substantial, direct and intellectual contribution to the work. They
1075 have read and agreed to the published version of the manuscript.

1076

1077

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1084

1085

1086 **CONFLIT OF INTEREST**

1087 The authors declare that this review was conducted in the absence of any commercial or
1088 financial relationships that could be construed as a potential conflict of interest.

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Figure 1. Immune cells and their respective locations in the CNS and PNS in ALS and FTD pathologies. Major therapeutic targets are represented on the different cell types. The expression of the different targets was indexed through researches with proteinatlas.org.

COX-2: Cyclooxygenase-2; CSF1R: Colony stimulating factor 1 receptor; IL-1R1: Interleukin-1 receptor 1; IL-6R: Interleukin-6 receptor; NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells; NRF2: Nuclear factor erythroid-2-related factor 2; PDE: Phosphodiesterase; PGE2: Prostaglandin E2; PI3K: Phosphoinositide 3-kinase; PPARγ: Peroxisome proliferator-activated receptor gamma; ROCK: Rho-associated protein kinase; SIP: Sphingosine-1-phosphate receptor; TKR: Tyrosin-kinase receptor; TLR-4: Toll like receptor 4.

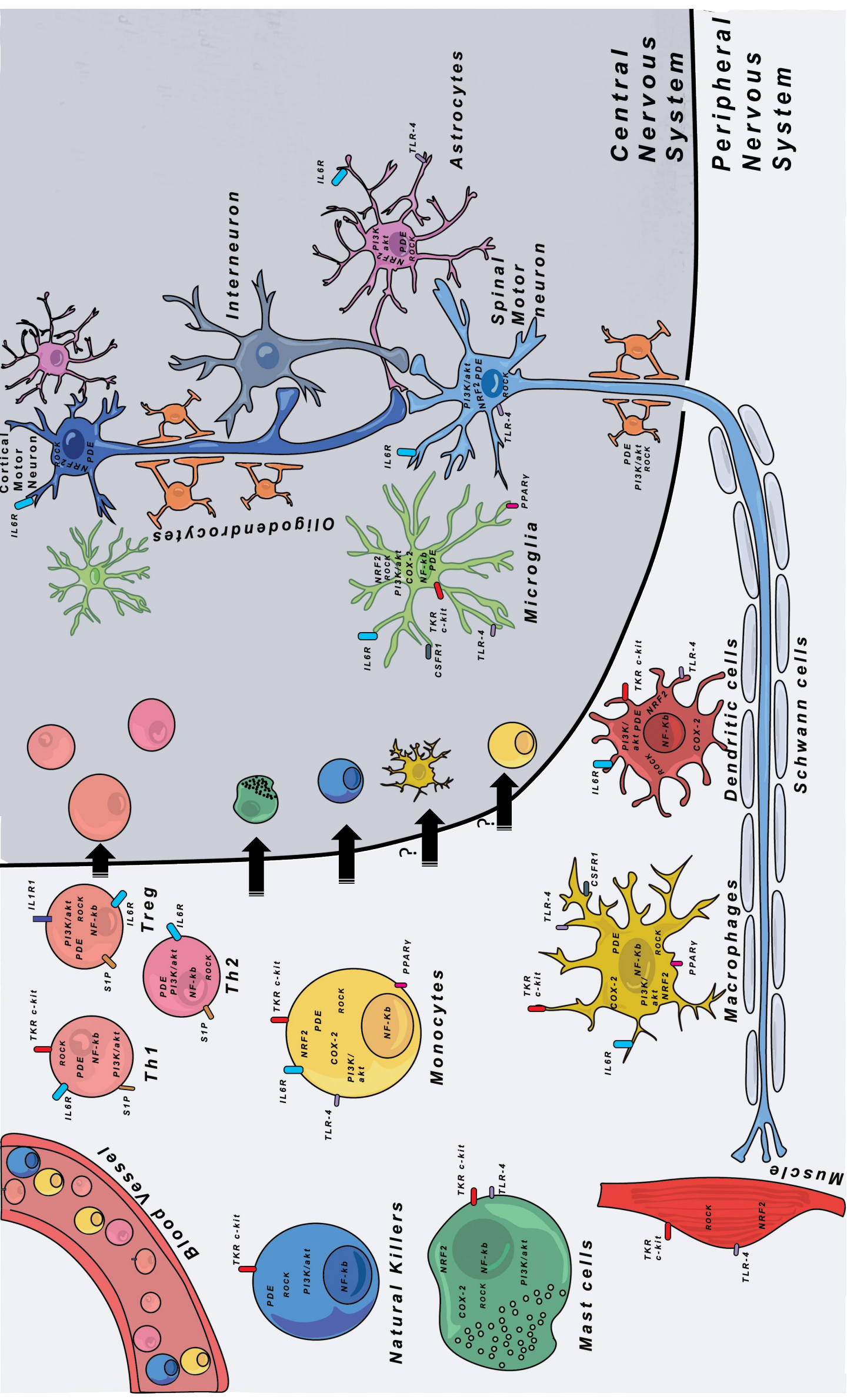


Figure 2. Main immune modulatory molecules tested in ALS clinical trials. On the left are presented the different molecules with their corresponding colors. On the figure each color dot next to the different cell types shows where the immune modulatory drug is supposed to have an effect. Some cell types targeted by the drugs were well studied in the literature (see table), but in some cases we also completed the data with theoretical targets depending on the expression of the target on specific cell types indexed with proteinatlas.org, and for which no information in the literature was found. *COX-2*: Cyclooxygenase-2; *CSF1R*: Colony stimulating factor 1 receptor; *IL-1R1*: Interleukin 1 receptor 1; *IL-2*: Interleukin-2; *IL-6R*: Interleukin 6 receptor; *NF-kB*: Nuclear factor kappa-light-chain-enhancer of activated B cells; *NRF2*: Nuclear factor erythroid-2-related factor 2; *PDE*: Phosphodiesterase; *PGE2*: Prostaglandin E2; *PI3K*: Phosphoinositide 3-kinase; *PPAR γ* : Peroxisome proliferator-activated receptor gamma; *ROCK*: Rho-associated protein kinase; *S1P*: Sphingosine-1-phosphate receptor; *TKR*: Tyrosin-kinase receptor; *TLR-4*: Toll like receptor 4.

- Minocycline
- Thalidomide
- Pioglitazone
- Fingolimod Phosphate
- Dimethyl Fumarate
- Glatiramer Acetate
- Anakinra
- Tocilizumab
- NP001
- Celecoxib
- Low dose IL-2
- Rapamycin
- Ravulizumab
- RNS60
- Masitinib
- Ibudilast
- Zilucoplan
- Fasudil
- Verdiperstat

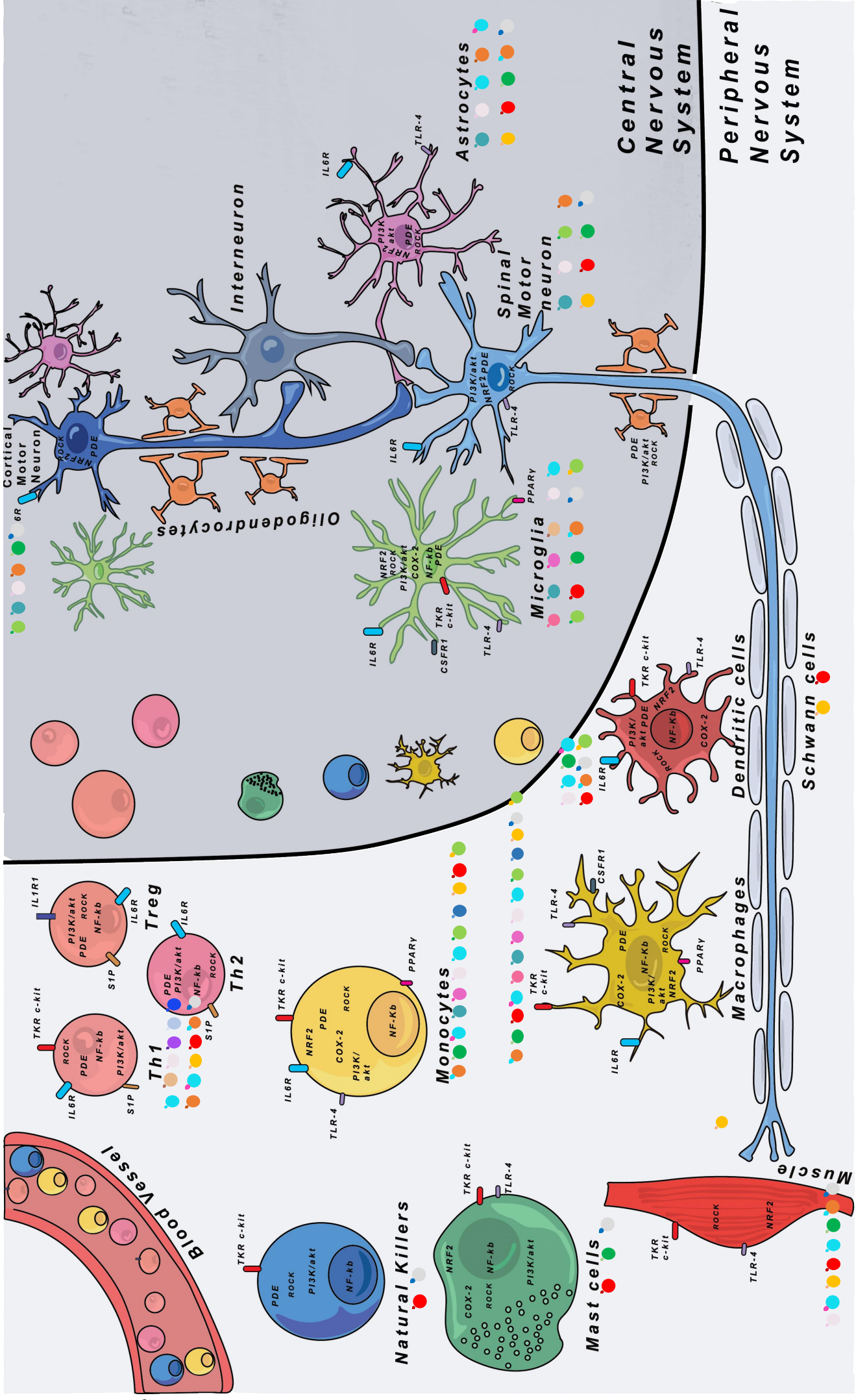


Table 1. Summary of publications studying circulating inflammatory molecules in ALS and FTD pathologies. This table recapitulates the circulating inflammatory molecules found in patients, that can be secreted by several immune cell types. *8-OHdG*: 8-hydroxydesoxyguanosine; *bFGF*: basic fibroblast growth factor; *CCL5*: C-C motif chemokine ligand 3; *CCL11*: C-C motif chemokine ligand 11; *CCL26*: C-C motif chemokine ligand 26; *CXC5R*: C-X-C motif chemokine receptor 5; *FasL*: Fas ligand; *FGF-2*: Fibroblast growth factor 2; *G-CSF*: Granulocyte-colony stimulating factor; *GM-CSF*: Granulocyte-macrophage colony-stimulating factor; *GSH*: glutathione; *GSSG*: Glutathion disulfure; *IFN- γ* : Interferon-gamma; *IL-1 β* : Interleukin-1 beta; *IL-2*: Interleukin-2; *IL-4*: Interleukin-4; *IL-5*: Interleukin-5; *IL-6*: Interleukin-6; *IL-7*: Interleukin-7; *IL-8*: Interleukin-8; *IL-9*: Interleukin-9; *IL-10*: Interleukin-10; *IL-10RA*: Interleukin 10 receptor subunit alpha; *IL-11*: Interleukin-11; *IL-12*: Interleukin-12; *IL-12p70*: Interleukin-12p70; *IL-13*: Interleukin-13; *IL-15*: Interleukin-15; *IL-17*: Interleukin-17; *IL-17A*: Interleukin-17A; *IL-18*: Interleukin-18; *IL-23*: Interleukin-23; *IP-10*: Interferon gamma-induced protein 10; *LPC*: Lysophosphatidylcholine; *MCP-1*: Monocyte chemoattractant protein 1; *MDA*: Malondialdehyde; *MIP-1 α* : Macrophage inflammatory protein-1 alpha; *MIP-1 β* : Macrophage inflammatory protein-1 beta; *MMP9*: Matrix metalloproteinase 9; *PAF*: Platelet-activating factor; *PDGF-BB*: Platelet-derived growth factor- BB; *RANTES*: Regulated Upon Activation, Normally T-Expressed, And Presumably Secreted (or *CCL5*); *TGF- β 1*: Transforming growth factor beta 1; *TGF- β 2*: Transforming growth factor beta 2; *TIMP2*: Tissue inhibitor of metalloproteinases 2; *TNF- α* : Tumor necrosis factor alpha; *TRAILR3*: tumor-necrosis-factor related apoptosis inducing ligand receptor 3; *VEGF*: Vascular endothelial growth factor; *YKL-40*: Chitinase 3-like 1.

| Model | Compartment | ALS | FTD |
|------------------------------------|--------------|---|---|
| Circulating inflammatory molecules | CNS | <p>Increased circulating factors in CSF: Cytokines and chemokines: IL-2, IL-4, IL-5, IL-7, IL-8, IL-9, IL-12, IL-15, IL-17, IFN-γ, TNF-α, eotaxin, CCL11, MIP-1α, MIP-1β, MCP-1, IP-10 (Tanaka et al., 2006; Kuhle et al., 2009; Mitchell et al., 2009; Tateishi et al., 2010; Gonzalez-Garza et al., 2018); growth factors: FGF-2, VEGF, G-CSF, GM-CSF, PDGF-BB (Tanaka et al., 2006; Mitchell et al., 2009; Tateishi et al., 2010; Furukawa et al., 2015; Guo et al., 2017)</p> <p>Contradictory results: increase or decrease of IL-10 (Tanaka et al., 2006; Mitchell et al., 2009)</p> | <p>Increased circulating cytokines in the CSF: IL-8, IL-11, IL-23, MCP-1, IP-10, Eotaxin-3, TGF-β1, YKL40 (Sjögren et al., 2004; Galimberti et al., 2006; Galimberti et al., 2008; Hu et al., 2010; Bossù et al., 2011; Galimberti et al., 2015; Teunissen et al., 2016)</p> <p>Decreased circulating cytokines in the CSF: IL-12, IL-15, IL-17, TNF-α, RANTES (Hu et al., 2010; Rentzos et al., 2006a; Galimberti et al., 2015)</p> <p>Increased inflammatory lipids in the CSF: LPC, PAF (Phan et al., 2020)</p> |
| | Blood | <p>Increased circulating factors linked with extracellular matrix remodeling: MMP9, TIMP2, (Andrés-Benito et al., 2017); oxidative stress markers: GSSG, 8-OHdG, MDA (Blasco et al., 2017); circulating cytokines/chemokines: IL-1β, IL-4, IL-8, IL-12p70, IL-13, IL-15, IL-17A, IL-18, TNF-α, MIP-1α, MCP-1, eotaxin (Kuhle et al., 2009; Italiani et al., 2014; Ehrhart et al., 2015; Ngo et al., 2015; Lu et al., 2016; Guo et al., 2017; Blasco et al., 2017; Prado et al., 2018; Tortelli et al., 2020; Brodovitch et al., 2021); growth factors: G-CSF, GM-CSF, bFGF, VEGF (Guo et al., 2017)</p> <p>Downregulation of inflammatory cytokines chemokines CCL5, CXCR5, TGF-β2, IL-10RA (Ehrhart et al., 2015; Andrés-Benito et al., 2017); oxidative stress markers : GSH (Ehrhart et al., 2015; Blasco et al., 2017)</p> <p>Contradictory results: increase or decrease of IL-2, IL-5, IL-6, IL-10, IFN-γ, (Ehrhart et al., 2015; Lu et al., 2016; Andrés-Benito et al., 2017; Blasco et al., 2017; Guo et al., 2017; Prado et al., 2018; Tortelli et al., 2020; Brodovitch et al., 2021)</p> | <p>Increased peripheral circulating cytokines IL-6, IL-8, IL-15, IL-17, CCL26, MCP-1, IP-10, TNF, FasL, TRAILR3 (Bossù et al., 2011; Miller et al., 2013 TDP-43; Gibbons et al., 2015)</p> <p>Decreased peripheral circulating cytokines IL-1α, IL-6, IL-12, IL-23, RANTES, TNF (Santos et al., 2014; Galimberti et al., 2015)</p> |

Table 2. CD4 T helper cell subsets, their molecular signatures and their main effector function in immunity. *FoxP3: Forkhead box P3; GATA-3: GATA Binding Protein 3; IFN- γ : Interferon-gamma; IL-2: Interleukin-2; IL-4: Interleukin-4; IL-5: Interleukin-5; IL-9: Interleukin-9; IL-10: Interleukin-10; IL-13: Interleukin-13; IL-17A: Interleukin-17A; IL-17F: Interleukin-17F; IL-21: Interleukin-21; IL-22: Interleukin-22; IL-31: Interleukin-31; RORa: Retinoic acid-related orphan receptor α ; ROR γ t: Retinoic acid-related orphan receptor gamma t; STAT1: Signal transducer and activator of transcription 1; STAT4: Signal transducer and activator of transcription 4; STAT6: Signal transducer and activator of transcription 6; T-bet: T-box expressed in T cells; TGF- β : Transforming growth factor beta; Th: T helper cell; TNF- α : Tumor necrosis factor alpha; TNF- β : Tumor necrosis factor beta.*

| CD4 T subsets | Master transcription factors | Secreted cytokines | Effector functions in adaptive immunity |
|---------------|--------------------------------|---|---|
| Th1 | T-bet STAT4 STAT1 | IFN-γ IL-2 TNF- α TNF- β | Clearance of intracellular pathogens (e.g. viruses) by cell mediated immunity such as CD8 T cell and macrophage activation (Mosmann et al., 1989) |
| Th2 | GATA-3 STAT6 | IL-4 IL-5 IL-9 IL-13 IL-31 | Clearance of extracellular pathogens and activation of antibody-producing B cells (humoral immunity) (Zhu et al., 2006) |
| Th17 | ROR γ t ROR α | IL-17A IL-17F IL-21 IL-22 | Clearance of extracellular pathogens (mainly at mucosal and epithelial surfaces) Antimicrobial properties against (Liang et al., 2006) Recruitment of neutrophils (Laan et al., 1999) |
| Treg | FoxP3 | IL-10 TGF-β | Immune tolerance Anti-inflammatory function (von Boehmer et al., 2005) |

Table 3. Summary of publications studying immune cell involvement in ALS and FTD pathologies. This table recapitulates what is known about dysfunction of immune cells in ALS and FTD, in both human and animal models. Mast cells, monocytes, phagocytes (macrophages and microglia) and NK cells belong to innate immunity. T cells (CD4 and CD8 T cells) and B cells belong to adaptive immunity. Dendritic cells make the link between innate and adaptive immunities. *ALS: Amyotrophic lateral sclerosis; C9orf72: Chromosome 9 open reading frame 72; CCL3: C-C motif chemokine ligand 3; CCR2: C-C motif chemokine receptor 2; CD4: Cluster of differentiation 4; CD8: Cluster of differentiation 8; CD14: Cluster of differentiation 14; CD16: Cluster of differentiation 16; CD68: Cluster of differentiation 68; CNS: Central nervous system; CSF: Cerebrospinal fluid; CTLA-4: Cytotoxic T-lymphocyte associated protein 4; fALS: familial amyotrophic lateral sclerosis; FoxP3: Forkhead box P3; FTD: Frontotemporal dementia; FTL D: Frontotemporal lobar degeneration; Gata-3: Gata Binding Protein 3; GRN: Granulin precursor; ICAM1: Intercellular adhesion molecule 1; IFN- γ : Interferon-gamma; INPP5D: Inositol polyphosphate-5-phosphatase D; ITGB2: Integrin subunit beta 2; PNS: Peripheral nervous system; sALS: sporadic amyotrophic lateral sclerosis; SELL: Selectin L; SOD1: Superoxide dismutase 1; TDP-43: Transactive response DNA binding protein 43; Th: T helper cell; Treg: Regulatory T cell.*

Table 4. List of the different immune modulatory therapeutical approaches which entered clinical trials. Main targets are listed for each molecule, which may be non-exhaustive as some molecules have very large spectrum of action which is still not well described. The main effects are presented similarly. The listing is based on actual literature and may evolve with time. Targeted cell types are presented. In blue are the cell types predicted to be targeted by the molecule in accordance with the literature. In black are other cell types expressing the target and that may also be impacted by the therapeutic. The expression of each cellular target was investigated via proteatlas.org in order to index cell types susceptible to be targeted by the drug. Main focus was driven on immune related cell types. Trials are sorted depending on their clinical trial ongoing status. *3' UTR: 3' Untranslated Transcribed Region; ALS: Amyotrophic lateral sclerosis; ALSAQ-40: Amyotrophic lateral sclerosis assessment questionnaire; ALSFRS-R: Amyotrophic lateral sclerosis functional rating scale; Apaf-1: Apoptotic protease activating factor 1; BDNF: Brain-derived neurotrophic factor; C5: Complement 5; CNS: Central nervous system; CSF: Central nervous system; COX-2: Cyclooxygenase-2; CSF1R: Colony stimulating factor 1 receptor; DC: Dendritic cell; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; IL-1 α : Interleukin-1 alpha; IL-1 β : Interleukin-1 beta; IL-1R: Interleukin-1 receptor; IL-2R: Interleukin-2 receptor; IL-4: Interleukin-4; IL-6: Interleukin-6; IL-6R: Interleukin-6 receptor; IL-10: Interleukin-10; IL-12: Interleukin-12; IL-15R: Interleukin-15 receptor beta; iNOS: Inducible nitric oxide synthase; MAPK: Mitogen-activated protein kinase; MBP: Myelin basic protein; MG: Microglia; MIF: Macrophages migration inhibitory factor; MP: Macrophage; MPO: Myeloperoxidase; mTOR: mechanistic target of rapamycin; NF- κ B: Nuclear factor kappa-light-chain-enhancer of activated B cells; NFL: Neurofilament light chain; NGF: Nerve growth factor; NK: Natural killer; NMJ: Neuromuscular junction; NO: Nitric oxide; NRF2: Nuclear factor erythroid-2-related factor 2; NT-4: Neurotrophin 4; NTF: Neurotrophic factors; PDE: Phosphodiesterase; PGE2: Prostaglandin E2; PPAR γ : Peroxisome proliferator-activated receptor gamma; ROS: Reactive oxygen species; SIP: Sphingosine-1-phosphate receptor ; SIP1: Sphingosine-1-phosphate receptor 1; TLR-4: Toll like receptor 4; TNF α : Tumor necrosis alpha; TKR: Tyrosin-kinase receptor; VFC: Vital force capacity.*

Appendice 2

In preparation

Neurofilament accumulations in motor neurons of patients with Amyotrophic lateral Sclerosis impair structural axonal initial segment plasticity

(title: max 15 words)

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Introduction

Amyotrophic Lateral Sclerosis (ALS) is a degenerative disorder that primarily affects the motor system. It is the most common motor neuron (MN) disease in adults with a pathology characterized by a progressive degeneration of pyramidal tracts controlling movements. The disease is heterogeneous with various clinical onsets and different rates of progression. Moreover, whereas dozens of causing and associated genes were identified, most ALS cases are sporadic. The pathology is very aggressive with a survival time limited to 2-5 years after disease onset and there is still no treatment so far able to interrupt disease progression¹. Decades of research highlighted that many mechanisms could be part of the degenerative process in MNs, with three main hallmarks deserving today more attention: the role of protein inclusions, excitability, and axonal transport defects².

Protein inclusions were described in MNs of post-mortem tissues^{3,4,5,6} of ALS patients carrying or not mutations in identified causing genes. Most of the time, inclusions contained normal and/or mutant proteins encoded by causal ALS genes including TDP-43 (TAR DNA-binding protein), SOD-1 (superoxide dismutase 1), FUS (fused-in-sarcoma), dipeptide repeats (DPRs) encoded by hexanucleotide repeats in C9ORF72, NFEH (heavy neurofilament), SQSTM1 (p62), PFN1 (Profilin1), PRPH (Peripherin), but also proteins encoded by non-ALS causal genes like CST3 (Cystatin C), and TRFE (Transferrin)^{3,4,5,7,8}. Data evidenced that different types of inclusions can form in cells and are made of heterogeneous contents that can differ from one ALS case to another. While some may be causal, others may be consequences of the chronic MN stress. Anyway, the formation of these inclusions have necessarily a progressive impact on proteome homeostasis and dysfunction of the proteome is strongly suggested to participate to MN degeneration⁹. The maintenance of proper MN homeostasis relies on the specific distribution of cargoes into their two morphologically and functionally distinct domains: the somatodendritic and the axonal compartments. For example, Neurofilaments (NFs) and presynaptic precursor vesicles are mainly distributed along axons, whereas lysosomes and mitochondria are present in both domains¹⁰. NFs as scaffolding proteins of the axonal cytoskeleton play important roles in the transport and docking of organelles. Disruption of NFs organization and expression or metabolism is characteristic of neurological syndromes including ALS¹¹. Another major structure involved in the neuron polarity is the proximal axonal region. This region includes the preaxonal exclusion zone or axon hillock (AH) and the axonal initial segment (AIS)¹². AH is the region where occurs recruitment of axonal cargoes and

exclusion of somatodendritic ones. At first, the AIS was identified as the structure that generates and shapes the action potential before its propagation along the axon^{12, 13, 14}. Then, AIS was shown to play an essential role in axon-dendritic polarity regulating protein mobility between the soma and the axon with a surface diffusion barrier^{15, 16}. This highly ordered specialized region includes ion channels and cell adhesion molecules anchored to a specialized ankyrinG/spectrin scaffold connected to both actin filaments and microtubules. Each neuron subtype seems to have a specific AIS responsible for its particular excitability properties, depending on AIS length, its distance from the soma, and its combination and distribution of voltage-gated sodium (Nav) and voltage-gated potassium (Kv) channel isoforms^{17, 18}. Recently, dysregulations of key components of the AIS were shown to be affected in several human pathological conditions^{19, 20} and current efforts are made to understand whether alteration of AIS plasticity affects the course of diseases¹⁵.

In the present study, we hypothesized that structural AIS plasticity alterations could be both cause and consequence of protein accumulations and axonal transport defects in ALS MNs and that NFs could play a major role in these alterations. Why? Firstly, ultrastructure studies done in the 90's on ALS patient's post-mortem tissues, far before AIS molecular description, showed axonal spheroids containing different types of vesicles, lysosomes, mitochondria, NFs, and microtubules². Swollen proximal axonal regions with preferential accumulations of NFs and mitochondria were described^{21, 22, 23, 24, 25, 26}. One of these ultrastructure reports also suggested that impairment of fast axonal transport in proximal axons was linked to organelles accumulation²⁷. Secondly, more recent ultrastructural studies in the SOD1^{G93A} mouse model showed that as early as at the presymptomatic stage, NFs and mitochondria accumulated in these proximal axonal regions^{28, 29}. Finally, in the last decade, NFs were detected in CSF and peripheral blood of ALS patients as a consequence of axonal degeneration and interestingly NFs release occurred at the same time as first pathological signs of the disease irrespective of the primary causal event³⁰.

Human induced pluripotent stem cells (iPSC) were generated and MNs were produced from patients carrying mutations in the three main genes responsible for ALS - *C9ORF72*, *SOD1* and *TARDBP* - as well as isogenic and non-related iPSC clones. Whereas ALS MNs survived as control ones, progressive neurite damages occurred in MNs. NFs accumulations, both the light chains (NF-L) and phosphorylated heavy chains (pNF-M/H), were observed at early time points of the differentiation kinetic. We showed that the latter could be localized in the proximal axonal regions of ALS iPSC-derived MNs and of patient's MNs in post-mortem tissues. In the presence

of pNF-M/H accumulations, significant delocalizations of the AIS and of sodium Nav channels were measured, as well as an increased of axonal calibers, showing AIS structural alterations. In parallel, ALS MNs acquired altered excitability with time in culture. Taken together, our findings reveal a novel pathway by which NFs disrupt AIS motoneuronal plasticity.

RESULTS

ALS MNs undergo slow degeneration processes.

In order to compare intrinsic MN defects in patients carrying different ALS mutations, iPSC clones were generated from four patients: one patient mutated in *SOD1*, two patients mutated in *C9ORF72*, and one patient mutated in *TARDBP* (**Supplementary Table 1**). The patient harboring the N139D mutation in the *SOD1* gene (*SOD1*^{N139D}) was reported previously³¹ and developed a slow progressive disease. The patient mutated in *TARDBP* (*TARDBP*^{G348C}) and the two patients harboring hexanucleotide expansions in the *C9ORF72* (*C9ORF72*^{(G4C2)ⁿ) gene developed rapid disease forms. These cases were reported previously. *SOD1*^{N139D} and *TARDBP*^{G348C} iPSC isogenic controls were generated using the CRISPR-CAS9 technology (*SOD1*-ISO and *TARDBP*-ISO) (see Methods). Four non-related age and sex-matched control iPSC clones were also included in our analysis (**Supplementary Table 1**). We established two Ctrl69 iPSC clones from a 69-year old subject. Ctrl33 and Ctrl11 were previously described³². Ctrl60 was obtained from a European Collection.}

iPSC were fully characterized at the molecular and functional levels (**Supplementary Fig.1-4**). Clones expressed human embryonic stem (ES) cell markers (**Supplementary Fig. 1a**) and endogenous pluripotency-associated genes were re-activated compared to parental fibroblasts (**Supplementary Fig. 1b**). iPSC pluripotency was shown by *in vitro* three-germ layer differentiation assays (**Supplementary Fig. 1c**). Genome integrity of each iPSC clone was shown by Illumina Human OmniExpress-24 SNP arrays. SNP deviations analysis in iPSC clones compared to their original pool of fibroblasts confirmed the parental identity of each iPSC clone and that they had not acquired large-scale copy number variations (**Supplementary Fig. 3**). Genotyping of *SOD1*^{N139D} and *TARDBP*^{G348C} genetic mutations in ALS clones confirmed the presence of mutations in ALS iPSC and their absence in ISO-*SOD1* and ISO-*TARDBP* clones. Repeat-primed PCR confirmed the presence of hexanucleotide expansions in the *C9ORF72* gene in parental fibroblasts, iPSC clones and MNs (**Supplementary Fig. 4**).

All control and ALS iPSC clones were differentiated into MNs^{33, 34, 35} (**Fig. 1a-1c**). Within 10 days, more than 90% of differentiated cells expressed the OLIG2 transcription factor (**Fig. 1d**), a specific marker of spinal MN progenitors (pMNs). Seven days later, post-mitotic neurons expressed ISLET1, a spinal MN marker (**Fig. 1e**). These young MNs (yMNs) were still immature with no expression of the Vesicular Acetylcholine Transporter (VACHT), whereas 18 days later (T1) mature iPSC-derived MNs (mMNs) expressed this marker (**Fig. 1f**). Importantly, control and ALS MNs acquired firing properties as revealed by whole-cell patch-clamp recordings (see below), showing the functionality of iPSC-derived MNs.

Quantifications of ISLET1 and VACHT positive neurons were performed at different time-points. **Figure 1g** shows percentages of ISLET1-positive neurons for each control and ALS iPSC clone at T0. No significant difference was measured between control and ALS clones (all controls (including SOD1-ISO and TARDBP-ISO): 79.1 ± 1.6 %; SOD1^{N139D}: 73.5 ± 2.3 %; C9ORF72^{(G4C2)ⁿ}: 84.8 ± 1.6 %; TARDBP^{G348C}: 83.2 ± 4.3 % (mean \pm SEM)). At T1, percentages of β 3tubulin-positive neurons expressing VACHT were similar between control and ALS iPSC-derived MNs (all controls: 97.4 ± 1.1 %; SOD1^{N139D}: 98.3 ± 1.75 %; C9ORF72^{(G4C2)ⁿ}: 94.8 ± 2.5 %; TARDBP^{G348C}: 98.8 ± 1.4 % (mean \pm SEM)) (**Fig. 1h**). Ratios of ISLET1-positive MNs between T1 and T0 were similar in control and ALS MN cultures (**Fig. 1i**). When lactate dehydrogenase (LDH) activities were measured as a marker of cell membrane damages, a significant slow progressive release of the cytosolic enzyme in supernatants of ALS MNs was measured (**Fig. 1j**). Taken together, these data showed that ALS progenitors and post-mitotic MNs carrying different ALS mutations were generated with the same kinetics and efficiencies compared to controls, survived similarly as control ones, but that ALS MNs underwent slow degeneration processes under basal and physiological conditions.

ALS and control iPSC differentiate into spinal cholinergic MNs of the lateral motor column.

In ALS patients, it is well established that the different MN subtypes are not equally affected^{36, 37} during disease progression. To identify MN subtypes generated in our cultures, we performed RNA-sequencing and transcriptome analysis of pMNs, yMNs and mMNs derived from control iPSC (clones 69-1; 69-2; 33) including isogenic iPSC (clones SOD1-ISO, TARDBP-ISO in pMNs and yMNs), and from different clones of the 4 ALS patients (clones SOD-1, SOD-2, TARDBP-1, TARDBP-2, C9ORF72-1.1, C9ORF72-2.1) (**Supplementary Table 1**). Principal component analysis (PCA) showed efficient segregations of cells according to their maturation

status (**Fig. 2a**). SOD1^{N139D}, C9ORF72^{(G4C2)ⁿ}, TARDBP^{G348C} and control MNs shared same RNA expression patterns of differentiation markers at the three stages (**Fig. 2b-2m**). Firstly, all control and ALS pMNs expressed markers of progenitors, OLIG2, PAX6, and NKX6.1, while ISLET1 and HB9 were expressed after differentiation in yMNs and mMNs (**Fig. 2b-2e, Supplementary Fig. 5a**). Importantly, markers of V1, V2 and V3 spinal interneurons (expressing Engrailed 1, CHX10 and NKX2.2 transcription factors, respectively) were not detected in differentiated MNs (**Supplementary Fig. 5b**). Secondly, whereas acetylcholinesterase (ACHE) expression increased with differentiation (**Fig. 2f**), no significant expression of genes encoding enzymes involved in the synthesis of different neurotransmitters was measured (**Supplementary Fig. 5c**), strongly suggesting that only cholinergic neurons were produced. VACHT expression at protein levels (**Fig. 1f**) and RNA levels (**Fig. 2g**) confirmed these results. Thirdly, cultures were devoid of glial cells, as assessed by immunofluorescent labelling (not shown) and the absent expression of mRNAs encoding GFAP (glial fibrillary acidic protein) and MBP (myelin basic protein) (**Fig. 2h and 2i**). Fourthly, MNs expressed a HOX code typical of the rostral spinal cord with high expression of HOXC456 and no expression of HOXC8 (**Fig. 2j and 2k**). Finally, we observed that LHX3 expression was negative in mMNs, while FOXP1 expression was detected in all control and ALS MNs (**Fig. 2l and 2m; Supplementary Fig. 5d**), showing that lateral motor column (LMC) MNs were produced. This global analysis showed that in our cultures iPS-derived MNs are the ones innervating voluntary limb muscles, those being primarily affected in non-bulbar onset ALS patients and thus being of particular interest to study ALS MN phenotypes.

Comparisons of transcriptome profiles between all ALS and control MNs suggest pathological roles of proteins involved in AIS formation and protein degradation.

The same transcriptomic analysis allowed us to ask whether ALS MNs harbored RNA expression alterations compared to control MNs. We looked for common differentially expressed genes between all ALS MNs and control MNs (fold change ≥ 1.5 and adjusted P-Value ≤ 0.05) at the three stages of MN differentiation. With these strength conditions of analysis - adjusted P-Value instead of unadjusted P-Value - no gene in ALS pMNs and ALS yMNs was significantly deregulated (**Supplementary Table 2**), suggesting that at these early time-points ALS MNs were similar to control MNs at least at the transcription level. In more mature MNs, we found 7 up-regulated genes in ALS MNs, with 4 of them belonging to specific

altered GO terms (**Table 1**). The most two DEG genes encode the cell adhesion molecule L1 like protein (CHL1) and cathepsin F (CTSF), two proteins of particular interest. Their increased expressions were confirmed by quantitative RT-PCR in ALS MNs (**Supplementary Figure 6**). CHL1 is a cell adhesion molecule that recruits Ankyrin G (AnkG), the central component of AIS¹², to the plasma membrane³⁸. Moreover, CHL1 was shown to guide regrowth of regenerating MN axons and to be involved in regulating cholinergic synapse formation³⁹. Cathepsin F is a lysosomal cysteine protease implicated in intracellular degradation and turnover of proteins^{40, 41, 42, 43}. Other up-regulated genes (**Table 1**) include RIMBP2 (RIMS binding protein 2) involved in synaptic transmission⁴⁴, NNAT (neuronatin) with a poorly understood physiological role but which may play a role as a regulatory subunit of ion channels^{45, 46} and HSPA1L (heat shock 70 kDa protein 1L), encoding a protein able to stabilize proteins against aggregation in conjunction with other HSPs⁴⁷. Taken together, these data suggested that common alterations between the different ALS forms involved responses to protein aggregations and AIS alterations.

Progressive alteration of excitability in ALS MNs.

As the AIS is directly involved in action potential initiations¹² and altered excitability is a common phenomenon reported in ALS patients^{48, 49}, we investigated electrophysiological properties of iPSC-derived MNs with whole cell patch-clamp recordings at two time-points (**Fig1a**; T2 and T3). Previous reports, despite discrepancy results between them, showed excitability changes in ALS iPSC-derived MNs⁵⁰ carrying different genetic mutations. In our experiments, before T2, MNs were still immature according to firing properties. Spontaneous action potentials (AP) were rarely recorded at any time point. Quantification and classification of recorded MNs according to their firing patterns in response to injected currents showed four different patterns (**Fig. 3a**) with a vast majority of MNs able to fire at least one AP (**Fig. 3b**). Whereas no significant differences were found between T2 and T3 in the percentages of control MNs able to fire one of the 4 AP firing patterns, we measured significant increases in percentages of AP with adaptive firing patterns in SOD1^{N139D} and C9ORF72^{(G4C2)ⁿ} MNs between T2 and T3 (**Fig. 3b**). The latter showed also significant decreases with time of their RMP (resting membrane potential) and AP half width, compared to control, SOD1^{N139D} and TARDBP^{G348C} MNs (**Fig. 3c and 3d**). Whereas AP thresholds were slightly increased between T2 and T3 in control and C9ORF72^{(G4C2)ⁿ}, they remained stable in SOD1^{N139D} or with a decreased trend in TARDBP^{G348C} MNs ($p = 0.0671$) (**Fig. 3e**). Rheobase, maximum frequency,

delay of first spike, peak amplitude and AP amplitude remained stable between T2 and T3 for ALS and control MNs (**Fig. 3f, 3g and Supplementary Fig. 7**). In the voltage-clamp mode, the recorded inward current had the fast activation and inactivation characteristics of voltage gated sodium (Na) channel, when the outward current had the slow activation and no inactivation of the voltage-gated potassium (K) channel. Interestingly, recordings showed that I_{Na}/I_K ratio significantly increased with time in SOD1^{N139D} and C9ORF72^{(G4C2)ⁿ} MNs, while the ratio had a trend to decrease in TARDBP^{G348C} MNs (**Fig. 3h**). Analysis of Na and K current densities showed alterations of sodium current densities in ALS MNs (**Fig. 3i and 3j**). Taken together, these results strongly suggested progressive alterations of MN excitabilities in the different ALS forms compared to controls, occurring more or less rapidly with maturation.

Early neurofilament accumulations in the soma and the axonal proximal region in ALS iPSC-derived MNs.

Abnormal protein accumulations forming inclusions are hallmarks of the ALS pathology and were described in ALS post-mortem tissues^{3, 4, 5, 6}. Another important hallmark reported in post-mortem tissues of ALS patients is neurite pathology and accumulation of NFs^{24, 25, 51} (Fisher 2004? A trouver). As these observations were done on electron microscopic images without immunolabelling of NFs, we assessed whether and where the phosphorylated NF form of medium/heavy chains (pNF-M/H) were accumulated in MNs of ALS patients. Post-mortem spinal cord tissues were stained for pNF-M/H (patients mutated in SOD1^{D83G}, C9ORF72^{(G4C2)ⁿ} and TARDBP^{G348V}) (**Supplementary Table 2**). In all three patients, we observed MN cell body loss and irregular neurite calibers of remaining MNs in the ventral horn of the spinal cord, compared to sensitive neurons in the dorsal horn (**Fig. 4a**). With higher magnification, we observed pNF-M/H inclusions, in tissues of all three patients, in soma and neurites of MNs, with more or less large and numerous neurite inclusions (**Fig. 4b-4d**). Interestingly, some positive beads were localized proximal to soma, in the AIS region.

In our cultures, we observed bead-like structures at T1 not only in SOD1^{N139D} iPSC-derived MNs but also in C9ORF72^{(G4C2)ⁿ} and TARDBP^{G348C} MNs (**Fig. 4e**). Immunostaining of NF-L, light chain subunits of NFs, showed both cell body accumulations and dashed neuritic stainings, in SOD1^{N139D}, C9ORF72^{(G4C2)ⁿ} and also in TARDBP^{G348C} MNs at T1 (**Fig. 4f**). Interestingly, at T0, NF-L accumulations were already observable and were more localized in cellular bodies of ALS MNs, while neurites were barely dashed with more intense NF-L staining intensities than controls (**Supplementary Fig. 8a**). Quantifications of somatic NF-L staining showed

significant accumulation in SOD1^{N139D} and C9ORF72^{(G4C2)ⁿ} MNs at T1 (**Fig. 4h**), while quantification was not significant for TARDBP^{G348C} MNs, despite evident accumulation of NF-L in both soma and neurites (**Fig. 4f and 4h**). For pNF-M/H, accumulations were concentrated in soma and were visible as soon as T0 (like NF-L) in all ALS forms (**Supplementary Fig. 8b**). At T1 specific accumulations in one proximal neuritic region, the AIS region (see below), were observed in SOD1^{N139D} and C9ORF72^{(G4C2)ⁿ} MNs (**Fig. 4g and 4i**).

Alterations of the structural integrity of proximal axonal region in ALS MNs.

The proximal axonal region of the MN includes two unique segments AH and AIS¹⁵. We analyzed whether the organization of this region and in particular the structure of the AIS - crucial for integration of synaptic inputs, initiation of AP as well as protein trafficking – was altered in ALS MNs presenting both excitability alterations and abnormal NF accumulations. First, we showed that pNF-M/H accumulations were exclusively in axons and not in dendrites. Co-immunostaining with antibodies against MAP2, AnkG (Ankyrin G) and pNF-M/H, to identify dendrites, the AIS and pNFs, respectively, showed that the AnkG staining was localized in MAP2-negative axons where pNF-M/H accumulated (**Fig. 5a**). Confocal analysis pointed that in pNF-M/H-positive MNs, the latter proteins accumulated at the AIS level (**Fig. 5b**). Obvious structural alterations of AIS in the presence of pNF-M/H are shown on figures 5b and 5c with larger AIS that can be split in its middle. The overlap of pNF-M/H and AnkG staining was partial and extended between the soma and the AIS. As the AH region has no known specific marker and thus cannot be specifically identified, and as its structural integrity seemed altered, we called the region between the soma and the AIS the “GAP”.

Next, to ask whether pNFs accumulations had an impact on the integrity of the axonal proximal region, we analyzed whether (i) the AIS was delocalized, (ii) voltage-gated sodium channels (panNav) distribution was altered, and (iii) axonal calibers were modified with a triple immunostaining (pNF-M/H, AnkG, panNav) (**Fig. 5c**). First we observed that soma areas (**Fig. 5d**) were not different between the different MN populations without pNF accumulations: control, mutant SOD1^{N139D}, C9ORF72^{(G4C2)ⁿ} and TARDBP^{G348C} MNs. However, in the presence of pNFs accumulations, we measured significantly smaller areas of SOD1^{N139D} MN somas as compared to control MNs. This difference was not observed for C9ORF72^{(G4C2)ⁿ} MNs. Next, AIS lengths were measured and we found no difference between the different MN populations with or without pNFs accumulations (**Fig. 5e**). Then, we analyzed the localization of pNFs accumulations and Nav channels distribution along the axon proximal region. To this

aim, pNF-M/H and panNav immunofluorescence integrated densities were measured between the soma and the beginning of the AIS (the GAP), as well as along the entire AIS. In SOD1^{N139D} MNs, pNFs accumulations were mostly localized closed to the soma (**Fig. 5f-5h**) - in the GAP region - whereas in mutant C9ORF72^{(G4C2)ⁿ} MNs pNFs accumulated in both the GAP and the AIS (**Fig. 5f-5h**). Nav channels were mostly localized in the AIS region and seemed equivalent in all ALS and control MNs (**Fig. 5i, 5j and Supplementary Fig. 9a**). However, ratios of panNav integrated density in the AIS over panNav integrated density in the GAP were significantly lower in the presence of pNFs accumulation, suggesting a modified distribution of Nav channels in pathological conditions (**Fig. 5k**). Next, we measured GAP lengths to look whether AIS localization was modified along the axon, especially when pNFs accumulated,. Interestingly, results showed very significant increases in GAP lengths in both mutant SOD1^{N139D} and C9ORF72^{(G4C2)ⁿ} MNs with pNFs accumulations compared to ALS MNs without accumulation or to control MNs (**Fig. 5l**), showing that mutant SOD1^{N139D} and C9ORF72^{(G4C2)ⁿ} MNs accumulating pNFs had an AIS segment delocalized towards a distal position when compared to the control MNs. Finally, to assess whether calibers of the proximal axonal regions were altered in the presence of pNFs accumulations, we measured mean caliber of both the GAP and the AIS (**Fig. 5m, 5n and Supplementary Fig. 9b and 9c**). Results showed that both regions of the axon had increased calibers in the presence of pNFs accumulations in mutant SOD1^{N139D} and C9ORF72^{(G4C2)ⁿ} MNs, suggesting specific alterations of these proximal axonal regions. Taken together, our data show that pNFs accumulations in the proximal region of axons altered the integrity of both AH and AIS with an AIS distal shift, a mis-localisation of Nav channels and increased calibers of the entire proximal axonal region in iPSCs-derived SOD1^{N139D} and C9ORF72^{(G4C2)ⁿ} MNs (**Fig. 5o**). Our results show for the first time a direct link between the presence of pNF-H accumulations and AH and AIS alterations in ALS MNs.

Discussion

In the present study we compared in a same experimental context cellular and molecular intrinsic defects in SOD1^{N139D}, C9ORF72^{(G4C2)ⁿ}, TARDBP^{G348C} and control iPSC-derived MNs. Importantly, we were able to show that our differentiation protocol allowed the generation of MNs that are the ones primarily affected in ALS patients. Whereas all ALS MNs survived as control ones, they progressively acquired excitability alterations and showed signs of degeneration with LDH leakage and accumulations of NF-L and pNF-M/H. The latter were

also observed in post-mortem tissues of ALS patients. These accumulations in the proximal regions in C9ORF72^{(G4C2)ⁿ} and SOD1^{N139D} MNs were directly linked to severe alterations of the structural organization of the AIS. These results expand our understanding of how NF accumulation can dysregulate components of the axonal cytoskeleton. In agreement, our transcriptomic analysis pointed to deregulations of genes involved in AIS formation and responses to protein aggregations in ALS MNs. Taken together, our results suggest that progressive protein accumulations and in particular of NFs result in structural and functional alterations of the axonal proximal region made of two sub-compartments crucial for polarity, transport and excitability of MNs.

Our transcriptomic analysis performed on iPSC-derived MNs allowed us to follow progenitor generation and characterize MN subtypes generated in cultures. We showed that mature MNs generated were (i) cholinergic, (ii) pure, without astrocytes, oligodendrocytes or proliferating precursors, (iii) had a rostral identity and (iv) expressed exclusively FOXP1, the marker of LMC MNs primarily affected in non-bulbar onset ALS patients^{52, 53}. To our knowledge our report is the first one that identify the MN subtypes present in their iPSC-differentiated cultures, assuring phenotypic analysis on MNs primarily affected in ALS patients. This is an important step forward in the field of ALS modeling with human iPSC, as previously published reports did not assess MN subtypes they generated^{50, 54}. Generation and analysis of both vulnerable and resistant MNs in a unique culture without distinction could mask some phenotypic differences. The mechanisms underlying the different MN death susceptibilities are not fully understood, but it is clear that the vulnerability of some MNs to degeneration is related to anatomical specificity and their transcriptome profile^{37, 55, 56} and that future analysis have to study specific MN subtypes affected in the disease to strengthen interpretations of results.

The slow degenerative processes we observed in C9ORF72^{(G4C2)ⁿ}, SOD1^{N139D} and TARDBP^{G348C} MNs were accompanied by changes in excitability with time in culture. In agreement with most studies^{50, 54} and despite the heterogeneity of protocols used to generate MNs in the different studies⁵⁷, we observed that ALS MNs survived for a long time in cultures. ALS MN rapid death can occur but in particular conditions like in cultures containing glial cells, demonstrating the role of environmental cells in the disease³². Few reports studied ALS MNs generated with the same protocol as the one we used^{35, 58, 59}. In *Bhinge et al.*⁵⁸ the death of SOD1^{E110G} MNs was detected after ~20 days of maturation. However, they cultured MNs at lower densities and used lower neurotrophic factor concentrations, thus creating stress conditions favoring MN death. Thereby in most studies, including ours, early observed

phenotypic defects were related to neurite alterations (e.g. neurite bead-like structures, tubulin fragmentation, reduced neurite growth), LDH leakage, and abnormal protein aggregates. Fujimori et al.⁶⁰ used precisely this set of features to show that MNs affected with mutations in SOD1, TARDBP and FUS, as well as MNs of sporadic ALS cases, could be distinguish ones from the others by a specific time-course of appearance of these different phenotypes. About protein aggregates, previous studies of ALS iPSC-derived neurons focused their attention on the specific accumulations of the protein linked to the studied mutated gene (SOD1 in MNs mutant in SOD1, TDP-43 in MNs mutated in TARDBP, etc)^{50, 54} and there is currently no study looking systematically to accumulations of the different proteins already described in tissues of patients. In our experiments, few SOD1 inclusions were observed only in neurites of SOD1^{N139D} MNs (**Supplementary Figure 10a**), while TDP-43 (phosphorylated or not) protein accumulations were observed only in few TARDBP^{G348C} MNs (**Supplementary Figure 10b, 10c**). Our data are in agreement with human studies that evidenced, even if a major common protein found in patient's aggregates is TDP-43, that inclusions were constituted of heterogeneous contents from one ALS case to another. Besides these inclusions, a common feature observed in all ALS forms is the involvement of NFs. Indeed, more than 50 years ago, electron microscopy revealed the accumulation of NFs in proximal axons of spinal MNs in post-mortem tissues of ALS patients^{24, 25, 51}. However for long NFs were seen as markers of late disease stages and did not get much interest. The renewed attention to NFs occurred when they were identified as sensitive biomarkers of neurodegeneration in ALS³⁰. Also, early NFs abnormalities were described in the SOD^{G93A} mouse model⁶¹, in iPSC-derived MNs mutated in TARDBP^{62, 63, 64} and SOD1⁶⁵, and we also showed similar phenotypes in SOD1^{N139D} and TARDBP^{G348C}, as well as in C9ORF72^{(G4C2)ⁿ} MNs. Thus, all these findings suggest a central role of NFs in the initiation of the pathological mechanisms. Also, with cumulating evidences that NFs are sensitive biomarkers of many neurodegenerative disorders and that their alterations are a major hallmark of these disorders, the hypothesis that emerges is that NF abnormalities could be a common early phenotype whatever the type of neurons affected in a neurodegenerative disease and that subsequent alteration are dependent on other factors including the neuron subtype.

Besides these phenotypes, several ALS iPSC-modeling studies, including ours, looked at excitability and performed MN electrophysiological recordings. Studies showed a large spectrum of altered excitability of ALS MNs⁵⁰. Whereas contradictory results were observed, this may due, at least partially, to protocol heterogeneities (e.g. MN differentiation protocols, cultures containing glial cells or not, time of recordings), but also this may reflect a continuum

from hyperexcitability to impaired firing as depolarization block occurs, instead of hyperexcitability and apparent hypoexcitability (resulting from prolonged hyperexcitability)^{50, 66, 67}. Human neurophysiological studies strongly supported such alterations in ALS patients⁴⁸ with imbalances between axonal potassium and sodium currents^{49, 68, 69, 70}. Recently *Howells et al.*⁷¹ compared nerve excitability properties of motor axons from ALS patients with those of control subjects. Their mathematical model suggested that changes in axonal excitability in ALS motor axons could be explained by a non-selective reduction in the expression of all ion channels and thus by a failure in the supply of ion channels and other membrane proteins from the diseased MNs. Interestingly, Huh et al.⁷², in their recent study of MN excitability at the pre-symptomatic phase in *SOD1^{G93A}* mice, showed that MNs were actively engaged in a highly homeostatic process to maintain their firing output and produce a normal motor behavior, and that MN degeneration in ALS constitutes a failure in cell homeostasis.

In MNs, cellular homeostasis relies on the specific distribution of cargoes in all the neuritic compartments and a unique feature of MNs that has implications for homeostasis is their incredibly long axons. The transport and distribution of cargoes (proteins, mRNA, lipids, organelles) all along the axon are a vital part ensuring energy supply and communications between soma and synaptic connections. The axonal transport of misfolded or aggregated proteins is also vital as the cell body is the primary site for degradative functions. During the past years, studies confirmed the crucial role of axonal transport in ALS MNs^{2, 73, 74} and highlighted links between axonal transport deficits and ALS pathogenesis in human tissues^{24, 25, 26} (Okada et al., 1995? To find...), in animal models^{75, 76, 77} and in iPSC derived-MNs^{50, 54} with mutations in *SOD1*⁷⁸, *C9ORF72*^{79, 80}, *FUS*³⁵ and *TARBDP*⁵⁹. In the same time, the AIS was identified as an essential highly structured segment with a central role in the axon-dendritic polarity and for protein diffusion between the soma and the axon^{15, 16}. In the present study we were able to show that the structure and organization of the proximal region was altered by pNF-M/H accumulations in *SOD1^{N139D}* and *C9ORF72^{(G4C2)ⁿ}* MNs. Moreover, at the molecular level, we showed the deregulated transcription of the *CHL1* gene that encodes a protein involved in the recruitment of AnkG to the plasma membrane³⁸. *CHL1* is a cell adhesion molecule playing several roles both during development (for the regulation of neuronal differentiation, neuron survival, neurite outgrowth, axon guidance) and in the mature nervous system (for synaptic activity and plasticity) and its upregulation in ALS MNs may respond to the ongoing perturbation of MN homeostasis. Interestingly, two transcriptomic studies revealed that the gene encoding AnkG was deregulated in post-mortem spinal cord tissues of five patients

with sporadic ALS and two patients with familial ALS including one SOD1^{A4V} patient⁸¹, and more interestingly in human motor nerve diagnostic biopsies of eight sporadic ALS patients, at a time representing an early phase of the disease⁸². More globally, during the past decade, AIS plasticity and alteration of its components were implicated in neurodegenerative, psychiatric, and brain trauma diseases^{12, 15, 83, 84}. For examples (i) an association between Alzheimer's disease (AD) and AIS-related genes was reported⁸⁵, (ii) when they are in close proximity to A β plaques, AIS number and length decreased⁸⁶, and (iii) a tau mutant protein was shown to impaired structural AIS plasticity⁸⁷. Our study is the first one describing a link between AIS alteration and ALS pathology.

With our study, we propose a new model to explain progressive MN axonal degeneration in ALS pathology. Subsequent to progressive NF accumulations, molecular and structural alterations of AH and AIS of MNs occur as early features, like a vicious circle, leading to a loss of AIS and axonal integrity with functional alterations including excitability modifications and specific defects in axonal protein trafficking to synapses and NMJs.

These results suggest that one component of any therapy might include preservation of the AIS structure. Consistent with this idea, Schafer et al. showed that calpain inhibitors can preserve the AIS after ischemic injury⁸⁸. Whether this approach will also work for ALS remains to be determined. Many questions remain regarding factors and pathways involved in AIS functions, especially those involved in the control of trafficking in MNs. Their identification could lead to the development of new therapeutic strategies¹⁹ that could be of interest for ALS patients.

Methods

Neuropathology on ALS patient spinal cord sections. ALS patients were enrolled in a brain donation program declared to the Ministry of Research and Universities, as requested by french laws. An explicit consent was signed by the patient himself, or by the next of kin, in the name of the patient, in accordance with the French Bioethical Laws. Patients are described in the **Supplementary Table 3**. Three levels of spinal cord (cervical, thoracic, lumbar) were taken. Immunostainings with pNFs were performed after deparaffinisation of 5 μ m thick sections by an automatic slide stainer (Benchmark XT Ventana staining system). Slides were pre-treated at 95°C in either CC1 (pH 8) proprietary retrieval buffer (Ventana Medical Systems). Antibodies were targeted with a biotin-free detection system (Ventana Medical Systems ultraview universal DAB Detection Kit).

Human cells. To establish a fibroblast collection from ALS patients to generate iPSC, skin biopsies were proposed to french ALS patients, in collaboration with the French National Referral Center for ALS at the Pitié-Salpêtrière hospital (Paris, France) and the Institut Pasteur (project promoter) (Paris, France). The protocol was validated by the Agence Française de Sécurité Sanitaire des Produits de Santé (AFSSAPS) and a Comité de Protection des Personnes (CPP) (CPP Ile de France VI; protocol 2010-33-ALSCELL). Fibroblasts from the TARDBP gene mutated patient were obtained from the Erasmus Hospital (Brussels, Belgium) after informed consent of the patient and approval of an ethical committee (CPP Ile de France II, DC 2011-534). Patients signed explicit consents. For control subjects, skin fibroblast cultures were obtained from the Centre de Ressources Biologiques (CRB) of Lyon (France). A statement of all biological samples was made according to french laws formulated by the Research Ministry.

iPSC generation and characterization. For two control subjects (11 and 33 years-old) and the patient TARDBP mutated patient, reprogramming of fibroblasts into iPSC was done with integrative retroviral vectors as previously described⁸⁹, except that TARDBP mutant fibroblasts were reprogrammed with 5 vectors encoding OCT4, SOX2, KLF4, C-MYC and NANOG. Other fibroblasts (the control subject of 69 years-old and the three patients mutated in SOD1 and C9ORF72) were reprogrammed with integration-free CytoTune-iPS 2.0 Sendai Reprogramming Kit (ThermoFisher Scientific), as described previously³⁴. Briefly, after transduction of fibroblasts with vectors, fibroblasts were plated either onto irradiated mouse embryonic fibroblasts (iMEF) or on Geltrex (ThermoFisher Scientific)-coated plates in feeder-free conditions. iPSC-like clones were observed ~3 weeks after genes transfer. Clones were picked and expanded, and manually passaged every 5-7 days. After few passages, colonies were passaged with 0.5mM UltraPure™ EDTA pH 8.0 (ThermoFisher Scientific), and maintained in Essential 8 Medium (ThermoFisher Scientific) on Geltrex-coated plates. One control iPSC clone (named Ctrl160) was obtained from the European Collection of Authenticated Cell cultures (HipSci supplier) (UK) and is derived from fibroblasts of a 60-64 years-old man (catalogue number: 77650156).

For characterization, iPSC were cultured as Embryoid Bodies (EBs) for 10 days, plated on Geltrex and cultured for another 10 days in fibroblast medium (DMEM supplemented with 10% fetal bovine serum) to induce spontaneous differentiation. Pluripotency and differentiation potential of iPSC and their EB derivatives were analyzed by immunostaining and PCR with reverse transcription (RT-PCR), as described previously^{32, 89}. When integrative vectors were

used for reprogramming, quantitative RT-PCR was performed as described previously³² to confirm efficient repression of exogenously introduced genes (**Supplementary Fig. 2**). Genome integrity was assessed by Illumina Human OmniExpress-24 SNP array (300,000 markers) (Integrage, Evry, France) and analyzed using KaryoStudio and GenomeStudio softwares (Illumina). SNP deviations in the iPSC lines were compared to the original pool of fibroblasts. These analysis confirmed the identity of the iPSC clone compared to the original fibroblasts and that they had not acquired large-scale copy number variations, although a small number of small-scale indels were found (well below the level that would be detected by G-banding). Genotyping of *SOD1* and *TARDBP* mutations as well as analysis of hexanucleotide expansions in the *C9ORF72* gene were performed by PCR on genomic DNA isolated from fibroblasts, iPSC clones and derivatives by PCR amplification and sequencing⁹⁰.

Gene correction of iPSC. To correct point mutations in iPSC clones carrying *SOD1*^{N139D} and *TARDBP*^{G348C} mutated genes the CRISPR/CAS9 technology was used^{91, 92}. Briefly, iPSC cultured in STEMFLEX medium were dissociated as single cells with Accutase (ThermoFischer Scientific). One million of cells were then transfected (4D Nucleofector system, Core Unit AAF-1002B and X unit AAF-1002; Lonza, Switzerland) with a ribonucleoprotein (RNP) complex and the ssODN matrix for homologous recombination (ssODN (single stranded oligodesoxynucleotide) sequences: GCTAGCAGGATAACAGATGAGTTAAGGGGCCTCAGACTACATCCAAGGGAATGT TTATTGGGCGATCCCAATTACACCACAAGCtAAACGACTTCCAGCGTtTCCTGTCTT TGTACTTTC for *SOD1* ; GCCATGATGGCTGCCGCCAGGCAGCACTACAGAGCAGT TGGGGTATGATGGGCATGTTAGCCAGCCAGCAGAACCAGTCAgGCCCATCGGGTA AcAAAtCAAACCAAGGCAACATGCAG for *TARDBP*). The RNP complex was prepared with equimolar concentrations of crRNA (crRNA sequences: AGGAGACGCTGGAAGTCGTT for *SOD1*; TGGTTTTGGTTATTACCCGA for *TARDBP*) and tracrRNA-ATT0550 (200μM), with 500μg of CAS9 protein (Integrated DNA Technologies (IDT), Iowa, US). After transfection, all cells were plated and two days later ATT0550-positive cells were sorted by FACS (MoFlo Astrios, Beckman Coulter; CYTO-ICAN platform, ICAN Institute, Paris, France) and plated at low concentrations on petri dishes coated with Laminin521 (StemCell Technologies) in STEMFLEX medium supplemented with Clone R (StemCell Technologies). Seven to 10 days later, individual iPSc clones were picked and expanded in 96-wells plates. Each clone was duplicated for either DNA analysis or freezing. Between 100 and 300 clones were picked. Genetically engineered clones were screened after

DNA extraction by PCR amplification of the targeted genomic region and sequencing (primer for PCR: forward: GTAGTGATTACTTGACAGCCC, Reverse : TAATGTTTATCAGGATACATTTCTACAG for SOD1; forward: CTGGCTTTAGATAAATTAATGCT, Reverse : GCTGAATATACTCCACACTGAACA for TARDBP) (primers for sequences: GTAGTGATTACTTGACAGCCC for SOD1; GCCGAACCTAAGCACAATAG for TARDBP). Corrected clones (SOD1-ISO; TARDBP-ISO) were then amplified and characterized at the molecular level. Potential off-target sites with 1, 2 or 3 mismatches were screening by Sanger sequencing and we found no off-target mutations in the selected clones.

Differentiation of iPSC into MNs

MNs differentiation was performed as described by Maury et al.³³ with slight modifications. Briefly, iPSC clones were dissociated within StemPro™ Accutase™ (ThermoFisher Scientific) and $2 \cdot 10^5$ cells/ml were suspended into a Neuronal Basic Medium (NBM) composed of 1:1 DMEM/F-12/Neurobasal™ (ThermoFisher Scientific), N2 (ThermoFisher Scientific) and B27™ supplement minus vitamin A (ThermoFisher Scientific), L-Ascorbic acid (0.01 μM, Sigma), supplemented with SB431542 (20μM, Tocris Bioscience, Bristol, UK), LDN-193189 (0.2μM, Stemgent) and CHIR99021 (3μM, Stemgent). Embryonic Bodies (EBs) were formed within one day, and 2 days later the NBM was supplemented with retinoic acid (0.1μM, Sigma) and SAG smoothed ligand (0.5μM, Enzo Life Sciences, NY, USA) until day 14. CHIR99021 was removed at day 2, while SB431542 and LDN-193189 were removed at day 4. BDNF, GDNF (10ng/mL, Miltenyi Biotec) were added at day 7. At day 9, DAPT (10μM, Sigma) was added and EBs were dissociated the next day with Trypsin-EDTA (ThermoFisher Scientific) (pMNs, **Fig. 1a**). Dissociated single cells were seeded onto coverslips coated either with PEI (Polyethyleneimine) solution (2mg/ml, Sigma) or Polyornithine (100μg/ml, Sigma), and Laminin (20μg/ml, Sigma). Between day 14 and day 17, cells were incubated in NBM with DAPT, BDNF and GDNF. From day 18 (T0, **Fig. 1a**) and until the end of each experiment, DAPT was removed from NBM, while CNTF and IGF1 were added (10ng/mL, Miltenyi Biotec) (**Fig. 1a**).

Immunocytochemistry

Cells were washed in phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde (PFA) (Euromedex) for 10 minutes at room temperature (RT). For ion channels staining, cells

were fixed 10 minutes with PFA 1% (RT) and 10 minutes in Methanol at -20°C. Cells were then incubated in a blocking solution (PBS supplemented with 1% bovine serum albumin (BSA), 2% normal goat serum (NGS), 0.1% Triton-X100) for 1 hour (RT). Primary antibodies were added and cells were incubated either for 2 hours at RT or overnight at 4°C. Secondary antibodies were added for 1 hour at RT in PBS supplemented with BSA and NGS (**Supplementary Table 4 and 5**). Nuclei were stained with Hoechst H33342 (ThermoFisher Scientific). Slides were mounted with Fluoromount G (Southern Biotech). Optical sectioning was obtained using an Imager.M2 equipped with AxioCAM MRm, Apotom.2 module, HXP 120C light source, Objectives/NA : 20X/0.8; 40X/1.3; 63X/1.4. Confocal images were obtained with SP8 X WLL, DMI8 stand (Leica).

Electrophysiology

Whole-cell patch clamp recordings were performed on iPSC-derived MNs, derived from SOD1-2, C9ORF72-1, TARDBP-1 iPSC clones, as well as three control clones. Three days after pMNs dissociation and plating, neurons were incubated (90 minutes at 37°C) with lentiviral vectors carrying the cDNA encoding the Red Fluorescent Protein (RFP) under the control of the HB9 promoter^{32, 93}. Lentiviral vectors were prepared as previously described³². RFP expression was observed few days later.

Patch pipette were pulled from thin wall borosilicate glass capillaries (Harvard Apparatus) on a Zeitz DMZ puller and had a resistance of 2-3 MΩ when filled with the intracellular solution, containing 10 mM KCl, 130 mM K-gluconate, 2 mM MgCl₂, 0.1 mM CaCl₂, 4 mM ATP-Mg, 0.3 mM GTP, 10 mM Na Phosphocreatine, 1 mM EGTA, and 10 mM HEPES, adjusted to pH 7.2 with KOH. The extracellular solution contained 140 mM NaCl, 3 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 10 mM glucose and 10 mM HEPES adjusted to pH 7.3 with NaOH. Whole-cell recordings were performed using an Axopatch 200B (Molecular Devices). Data were low-pass filtered with an eight-pole Bessel filter at 2 kHz and then digitized at a sampling rate of 50 kHz with a Digidata 1440A analog-to-digital interface. Data were acquired with pClamp 10 software (Molecular Devices). Capacitance transients were cancelled out. Series resistance was typically between 5-7 MΩ and was compensated by at least 70% for voltage-clamp experiments. Only cells with an $R_s \leq 10\text{M}\Omega$ and a resting membrane potential (RMP) more hyperpolarized than -20mV were included in data analysis. Access resistance was monitored at the end of the experiment and the cells were discarded if the series resistance change by more than 20%. In current clamp mode, holding potentials were corrected for a calculated liquid junction potential

of 15 mV. Resting membrane potential was determined by averaging for 30 s of recording and afterward a holding current was used to clamp the resting membrane potential as close as possible of -70mV. Voltage and current clamp protocols are provided in the results section. Whole cell recordings were performed on control and experimental group neurons in parallel on the same day. Only neurons with visually identified expression of fluorescent reporters were targeted for patching. Electrophysiological recordings were carried out at room temperature (22-24°C). Data were analyzed off-line using Clampfit 10 software (Molecular Devices) and OriginPro (Origin Lab Corporation). The AP trace immediately above threshold was used to determine the delay of the first spike, AP threshold, AP amplitude, and AP half width. AP frequency was obtained by measuring the maximum number of spikes during the current steps protocol (1 s).

Image Analysis

The maximum intensity projection of Z stacks composed by 3 images was used to measure neurons characteristics with a semi-automatic Fiji Macro. Only cells with a soma-derived AIS were included in the quantitative analysis. First, the AnkG-labelled AIS and pNF-M/H accumulations were automatically detected with an intensity threshold. Then, MNs cell bodies, the GAP (from the soma to the AIS) and the AIS region were drawn by hand on the pNF-M/H- and panNav-labelled images. ROIs were registered, source images were reopened, and the background was subtracted. The following measures were done automatically: (1) soma, AIS and GAP areas, (2) GAP and AIS lengths, (3) AnkG, pNF-M/H, panNav immunofluorescence integrated density in GAP and AIS regions. The integrated density corresponds to the product of Area and Mean (immunofluorescence) Gray Value. GAP and AIS integrated densities were summed to obtain pNF-M/H and panNav distribution from the soma to the end of the AIS. To compare panNav integrated density within the GAP, cells without GAP region were removed. Mean axonal calibers were obtained by dividing GAP and AIS areas by their respective length. We assumed that differences between integrated densities were significant after Kruskal-Wallis with Dunn's test if the fold difference was up to 3.

To measure somatic NF accumulation, the maximum intensity projection of Z stacks composed by 3 images was used. First, MNs cell bodies were circled by hand on β -tubulin-labelled images and ROI registered. Second, intensity threshold was set up on NF random images of control MNs. Then, area fractions per cells were calculated.

RNA extraction and sequencing

Total RNA was extracted using the RNeasy Plus Micro kit (Qiagen) according to the manufacturer's protocol. All RNAs were analyzed for quantity and quality. RNA concentrations were determined with High Sensitivity RNA ScreenTape on TapeStation (Agilent). cDNA libraries were prepared using KAPA mRNA Hyper Prep kit (Roche) according to the manufacturer's protocol. Sequencing was performed on a NextSeq 500 (Illumina) by the iGeneSeq ICM internal platform.

RNA-Seq data analysis

RNA-Seq data analyses were performed by GenoSplice technology (www.genosplice.com). Sequencing, data quality, reads repartition (e.g., for potential ribosomal contamination), and insert size estimation are performed using FastQC, Picard-Tools, Samtools and rseqc. Reads are mapped using STARv2.4.0⁹⁴ on the hg19 Human genome assembly. Gene expression regulation study was performed as already described⁹⁵. Briefly, for each gene present in the Human FAST DB v2016_1_full annotations, reads aligning on constitutive regions (that are not prone to alternative splicing) were counted. Based on these read counts, normalization and differential gene expression were performed using DESeq2⁹⁶ on R (v.3.2.5). Only genes expressed in at least one of the two compared experimental conditions were further analyzed. Genes were considered as expressed if their rpkm value was greater than 98% the background rpkm value based on intergenic regions. Pathways analyses were considered statistically significant for p -values ≤ 0.05 . Significant differential expression was defined as adjusted p -values ≤ 0.05 (P-Adj) after false discovery rate correction and fold-changes ≥ 1.5 . Analysis for enriched GO terms were performed using DAVID Functional annotation Tool (v6.8). GO terms with more than 10 genes per term were taken into account.

Quantitative PCR

Reverse transcription of 1 μ g RNA into cDNA was performed with the SensiFAST cDNA synthesis kit (Bioline, France). Quantitative reverse transcription PCR was then performed in a Roche LightCycler480 using 100 ng of equivalent cDNA and the LightCycler probes master according to Roche procedures. Primers were from ThermoFisher Scientific: Hs00544069_m1, Hs00186901_m1 and Hs02786624-g1 for CHL1, CTSF and GAPDH, respectively.

Statistical analysis

A minimum of three independent experiments, considered as 3 differentiation batches from iPSC, was always performed. Statistical analysis was performed with GraphPad Prism 6. D'agostino & Pearson normality test was used to verify Gaussian distribution for large data. For data passing normality test one-way ANOVA with Tukey's multiple comparisons test was performed. For data not passing normality test, Mann-Whitney test or Kruskal-Wallis with Dunn's multiple comparisons test were used. When normality test was not possible Gaussian distribution was assumed. Differences in integrated density were validated if the threshold was up to 3. Stars or Small Phi represent the significance levels as follow */ $p < 0.05$; **/ $p < 0.01$, ***/ $p < 0.001$, ****/ $p < 0.0001$.

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Author contributions

C.L-O: provision of study material, collection, assembly of data, data analysis, manuscript writing

E.L.: provision of study material, collection and assembly of data, data analysis

C.D.: collection and assembly of data, data analysis

B.L.: collection and assembly of data, data analysis

S.B.: provision of study material (CELIS-iPS platform)

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G.T.: provision of study material, collection of data

G.B.: provision of study material, collection of data

C.N.: provision of study material (fibroblasts of patient)

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L.L.: provision of study material (fibroblasts of patients)

D.S.: provision of study material (human tissues), collection and assembly of data, data analysis

C.L.: data analysis and interpretation

S.M.: data analysis and interpretation

S. Bo.: data analysis and interpretation

D.B.: conception and design, financial support, provision of study material, collection and assembly of data, data analysis and interpretation, manuscript writing

Conflict of interest statement

None

Figure 1. Comparisons of differentiation and survival of MNs with different ALS forms.

(a) Schematic representation of the differentiation protocol. Different time-points of analysis are indicated (T0, T1, T2 and T3). CHIR (CHIR99021), SB (SB431542), LDN (LDN193189), RA (Retinoic Acid), SAG (Smoothed agonist), NTF (Neurotrophic factors), DAPT (γ secretase inhibitor), pMNs (MN progenitors), yMNs (young MNs), mMNs (mature MNs). (b, c) Phase contrast microscopy images of yMNs (b) and mMNs (c), showing neuron networks. (d, e) Representative images of β 3-tubulin-positive (TUJ1) cells co-expressing OLIG2 (d) or ISLET1 (e) at day -7 and T0, respectively. (f) At T1, MNs express the Vesicular Acetylcholine Transporter (VACHT) marker. Nuclei were stained with Hoescht H33342 (Ho). Scale bars: 20 μ m. (g, h) Quantifications of ISLET1-positive (g) at T0 and VACHT-positive (h) iPSC-derived neurons at T1, respectively (mean \pm SEM). n = number of independent experiments. (g) Controls (Ctrl11, Ctrl33, Ctrl60, Ctrl69-1, Ctrl69-2): n = 29; SOD1 (SOD1-1, SOD1-2, SOD1-3): n = 17; SOD1-ISO: n = 3; C9ORF72 (¹C9ORF72-1, ²C9ORF72-2, ²C9ORF72-3): n = 18; TARDBP (TARDBP-1, TARDBP-2, TARDBP-3): n = 14; TARDBP-ISO: n = 3. (h): Controls (Ctrl33, Ctrl69-1, Ctrl69-2, SOD-ISO, TARDBP-ISO): n = 8; SOD1 (SOD1-1, SOD1-2): n = 4; C9ORF72 (¹C9ORF72-1, ²C9ORF72-2): n = 6; TARDBP (TARDBP-1, TARDBP-3): n=5. (i) Ratio of percentages of ISLET1-positive neurons between T0 and T1 (mean \pm SEM). n = number of independent experiments. Controls (Ctrl33, Ctrl69-1, Ctrl69-2, SOD-ISO, TARDBP-ISO): n = 7; SOD1 (SOD1-1, SOD1-2): n = 4; C9ORF72 (¹C9ORF72-1, ²C9ORF72-2): n = 4; TARDBP (TARDBP-1): n=3. No statistical significance by Kruskal-Wallis with Dunn's test (G) and one-way ANOVA with Tukey's test (h,i). (j) Colorimetric measurement of LDH in culture supernatants from control and ALS MNs at different time points. n = 3. Two-ways Anova, mixed-effects analysis, with multiple comparisons (*p < 0.05).

Figure 2. Transcriptome profiling show that iPSC differentiate into spinal MNs of the lateral motor column.

RNA sequencing and transcriptome analysis were performed on iPSC-differentiated cultures (pMNs, yMNs and mMNs) from 3 different controls (2 or 3 clones per time point), 2 different SOD1^{N139D} clones (2 to 4 clones per time point), 2 C9ORF72^{(G4C2)ⁿ} (3 to 4 clones per time point) and 1 TARDBP^{G348C} (2 to 3 clones per time point). (a) Principal component analysis (PCA) shows a time-dependent segregation of yMNs, pMNs and mMNs samples. (b-i) Deseq2

normalized gene expressions in pMNs, yMNs and mMNs of (b) OLIG2, (c) PAX6 (d) ISLET1, (e) HB9, (f) acetylcholinesterase (ACHE), (g) Vesicular acetylcholine transporter (VACHT), (h) GFAP, (i) MBP, showing the synchronized generation of spinal MNs devoid of glial cells. (j, k) Deseq2 normalized gene expressions of HOX-C4, C5 and C6 genes, markers of the rostral spinal cord' MNs, and HOXC8, a marker of more caudal MNs. (l, m) yMNs and mMNs express the transcription factor FOXP1, a specific marker of the lateral motor column, but not the LHX3 gene encoding a transcription factor involved in specification of MNs located in the median motor column.

Figure 3. Alterations of electrophysiological properties in ALS motor neurons.

Whole-cell patch-clamp recordings of iPSC-derived MNs from control subjects and ALS patients at T2 and T3. Recordings were done on the largest RFP-positive neurons visualized after gene transfer with a lentiviral vector encoding RFP under the control of the HB9 MN-specific promoter. n = number of recorded MNs from 2 or 3 independent experiments. Controls (Ctrl11, Ctrl69-1, Ctrl69-2): T2, n = 51; T3, n = 74; SOD1-2: T2, n = 18; T3, n = 15; ¹C9ORF72-1: T2, n = 24; T3, n = 22; TARDBP-1: T2, n = 17; T3, n = 21. (a-g) Current-clamp recordings. (a) Four AP firing patterns were observed among iPSC-derived MNs in response to injected currents (step 1s, 5-100 pA): repetitive, adaptive, one spike, none. (b) Percentages of MNs per firing pattern at T2 and T3. White numbers represent percentages of firing MNs. Statistical significances are shown for the adaptive PA firing groups for SOD1^{N139D} and C9ORF72^{(G4C2)ⁿ} MNs by a Fisher's exact test between T2 and T3 (Adjusted P-value. *p <0.05). (c) Resting membrane potential (RMP), (d) AP half width, (e) AP threshold, (f) rheobase, (g) maximum frequency. (h-j) Voltage-clamp recordings. (h) Ratio of Na^{+(peak)} and K^{+(low)} current densities during voltage steps of various amplitudes. (i) Na^{+(peak)} current density. (j) K^{+(low)} current density. (c-j) Mann-Whitney test (*p <0.05; **p <0.01; ***p <0.001) between T2 and T3.

Figure 4. Neurofilament accumulations in ALS iPSc-derived MNs.

(a-d) pNF-M/H staining and hematoxylin counterstaining of ALS patients' spinal cord sections. (a) Low magnification images showing loss of MNs and irregular calibers of remaining MNs in the anterior horn of the spinal cord of a SOD1^{D83G} patient (top image) when compared to sensitive neurons of the posterior horn (bottom image)(100X). (b-d) Top and bottom images: high magnification images of spinal cord anterior horns of (g) a SOD1^{D83G} patient, (h) a patient

with C4G2 expansions and (i) a TARDBP^{G348V} patient. Arrows show different patterns of pNF stainings with more or less dense and large axonal beads and positive neurites proximal to soma especially in the SOD1^{D83G} patient (200X). **(e)** Representative phase contrast images of control, SOD1^{N139D}, C9ORF72^{(G4C2)ⁿ} and TARDBP^{G348C} iPSC-derived MNs at T1. Arrowheads in insets show bead-like structures in neurites (Scale bars: 10 μ m). Differences in densities of neurite networks are obvious between control MNs and ALS MNs. **(f,g)** Control and ALS iPSC-derived MNs were labelled at T1 with antibodies against (f) the light neurofilament subunit NF-L or (g) the phosphorylated medium/high neurofilament subunits pNF-M/H. Images show cytoplasmic accumulations (arrows), neuritic NF-L positive beads (arrowheads) and pNF-M/H proximal axon accumulations (arrows). Nuclei were stained with Hoescht H33342 (Ho). Scale bars: 10 μ m. **(h, i)** Quantifications of (h) somatic NF-L and (i) neuritic pNF-M/H staining in the different MN cultures show significant differences between controls and either SOD1^{N139D} or C9ORF72^{(G4C2)ⁿ} MNs (mean \pm SEM). Statistical significance by Mann-Whitney test (*p<0.05; ***p<0.001). n = number of independent experiments. For NF-L: Controls (Ctrl33, Ctrl69-1, Ctrl69-2): n = 8; SOD1 (SOD1-1, SOD1-2) n = 6; C9ORF72 (¹C9ORF72-1, ²C9ORF72-2) n = 6; TARDBP (TARDBP-1, TARDBP-3): n = 4; SOD1-ISO: n = 3; TARDBP-ISO: n = 3. For pNF-M/H: Controls (Ctrl33, Ctrl69-1, Ctrl69-2): n = 4; SOD1 (SOD1-1, SOD1-2) n = 4; C9ORF72 (¹C9ORF72-1, ²C9ORF72-2) n = 5; TARDBP (TARDBP-1, TARDBP-3) n=5; SOD1-ISO: n = 3; TARDBP-ISO: n = 3.

Figure 5. Alterations of the structural integrity of proximal axonal regions in SOD1^{N139D} and C9ORF72^{(G4C2)ⁿ} iPSC-derived MNs.

(a, b) Examples of C9ORF72^{(G4C2)ⁿ} MNs co-immunostained with antibodies against pNF-M/H, MAP2 and AnkG, to identify axonal pNFs accumulation (arrows), MAP2-positive dendrites and AnkG-positive AIS, respectively. Scale bars: 20 μ m. A confocal image of pNF-M/H accumulation is shown in (b). Nuclei were stained with Hoescht H33342 (Ho). Scale bars: 10 μ m. **(c)** Representative images of a SOD1^{N139D} MN with or without pNF-M/H accumulation (Acc.) and labelled with antibodies against pNF-M/H, AnkG, and panNav. Dotted lines indicate soma localizations and an arrow indicates pNF-M/H accumulation. **(d-n)** Graphs of different measures done on images acquired in cultures of control, SOD1^{N139D}, C9ORF72^{(G4C2)ⁿ} and TARDBP^{G348C} MN with or without pNF-M/H accumulations (See material and Methods). Scale bars: 10 μ m. **(d-e)** Graphs showing soma' areas and AIS lengths. Acc: pNF accumulation **(f-h)** pNF-M/H (pNF) integrated density measured (f) between the soma and the end of the AIS, (g)

in the GAP and (h) in the AIS regions. **(i-j)** PanNav integrated density measured (i) between the soma and the end of the AIS and (j) in the GAP. **(k)** Ratio of panNav integrated density in the AIS to the GAP. See also Supplementary Fig. S8a. **(l-n)** Graphs showing GAP length, GAP mean diameter and AIS mean diameter. Accumulation of pNF-M/H modifies AIS localization and mean axonal caliber (mean \pm SEM). Statistical significance shown are either between ALS and control MNs (*) or between MNs with or without accumulation (ϕ) by Kruskal-Wallis with Dunn's test (ϕ p<0.05, **p<0.01, ****/ ϕ ϕ ϕ p<0.0001). n = number of analyzed MNs from 3 independent experiments. Controls (Ctrl33, Ctrl69-2): n = 81; SOD1 (SOD1-1, SOD1-2): n = 72 (with acc.) / n = 68 (without acc.); C9ORF72 (¹C9ORF72-1, ²C9ORF72-2): n = 63 (with acc.) / n = 71 (without acc.). TARDBP (TARDBP-1, TARDBP-2): n = 64. **(o)** Schematic representation of pNF-M/H accumulation and consequences on AH and AIS. (Scheme based on images from Servier Medical Art (<http://smart.servier.com/>)).

Supplementary Figure 1. Characterization of iPSC clones.

(a) Control and ALS iPSC clones express human pluripotency-associated markers including TRA1-60, NANOG and SSEA4. Representative immuno-staining are shown for three ALS clones (SOD1-1, ¹C9ORF72-1, TARDBP-1) and one control clone (Ctrl69-1). Ho = Hoechst (H33342 labelling of nuclei). Scale bar: 100 μ m. **(b)** RT-PCR show that all iPSC clones express mRNAs encoding endogenous pluripotency-associated genes (OCT4, CRYPTO, hTERT, REX, DNMTB3, NANOG, c-MYC). Parental fibroblasts are shown. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) is the internal control for mRNA detection. **(c)** Efficient differentiation of iPSC clones into the three germ layers in vitro. EBs were grown for 10 days in suspension and transferred to coated coverslips for 10 more days. Patients and control EBs derived from each iPSC clone gave rise to cells of the ectoderm (NESTIN or MAP2), the mesoderm (SMA) and the endoderm (AFP) layers. Representative images are shown. Scale bars: 50 μ m.

Supplementary Figure 2. Silencing of retroviral vectors in mutant TARDBP clones.

Quantitative PCR after reverse transcription showed that gene expression of retroviral vector genomes coding for OCT4, SOX2, KLF4, c-MYC and NANOG were re-expressed in iPSC clones compared to fibroblasts (T-hFib, value set to 1). Expression levels are expressed relative to the amounts of the reference GAPDH mRNAs.

Supplementary Figure 3. SNP Illumina analysis.

Genome integrity was assessed by Illumina Human OmniExpress-24 SNP arrays. Results were analyzed using the GenomeStudio software (Illumina) comparing parental fibroblasts and related iPSC clones. Karyograms were produced using the KaryoStudio software (Illumina). Amplifications (green) and deletions (orange) are shown alongside the relevant chromosome.

Supplementary Figure 4. Analysis of mutations in ALS patients' cells.

(a, b) DNA direct sequencing of control and patient derived-iPSC confirms the presence of (a) the N139D mutation in the *SOD1* gene and (b) the G348C mutation in the *TARDBP* gene in patient's cells. **(c,d)** Repeat-primed PCR was used to detect expanded GGGGCC hexanucleotides. The reverse primer designed on the repeat sequence allowed the amplification of multiple fluorescent PCR products that were visualized using GeneMapper software v4.0. Profiles are shown for (c) parental Ctrl69 fibroblasts and the Ctrl69-1 iPSC clone, (d) fibroblasts from the 2 ¹C9ORF72^{(G4C2)ⁿ} and ²C9ORF72^{(G4C2)ⁿ} patients, the ¹C9ORF72-1 and ²C9ORF72-2 iPSC clones, and MNs derived from ¹C9ORF72-1 iPSC clone. **(e, f)** Expression of both mutant and normal *SOD1* (e) or *TARDBP* (f) alleles in MNs derived from isogenic clones and non-isogenic clones generated after CRISPR/CAS9.

Supplementary Figure 5. Deseq2 normalized gene expression of transcription factors and enzymes.

(a-b) Deseq2 normalized gene expression of **(a)** NKX6.1, Engrailed 1 (En1), CHX10 and NKX2.2. **(c)** Deseq2 normalized gene expression of enzymes involved in different neurotransmitter synthesis (Tyrosine Hydroxylase (TH), Glutamate Decarboxylase (GAD1), Glutaminase (GLS1), Dopamine β-Hydroxylase (DBH) and Tryptophan Hydroxylase (TPH)). The table lists the different enzymes and neuron subtypes expressing these enzymes. **(d)** Representative immuno-staining images of mMNs expressing FOXP1 (in green) and SMI32 (in red). Nuclei were stained with Hoescht H33342 (Ho). Scale bar: 50 μm. (Rabbit anti-FOXP1, Abcam16645; mouse anti-SMI32, Covance R-1000).

Supplementary Figure 6. CHL1 and CTSF expression in MNs. Quantitative analysis of CHL1 and CTSF mRNA levels in MNs derived from control, SOD1^{N139D}, C9ORF7^{(G4C2)ⁿ} and TARDBPG^{348C} MNs. (n=3).

Supplementary Figure 7. Additional parameters of current-clamp recordings.

Delay to the first peak, **(b)** peak amplitude, **(c)** Action Potential (AP) amplitude measured at T2 and T3 in iPSC-derived MNs. Controls (Ctrl11, Ctrl69-1, Ctrl69-2): T2, n=51, T3, n=74; SOD1-2: T2, n=18, T3, n=15 ; ¹C9ORF72-1: T2, n=24, T3, n=22 ; TARDBP-1: T2, n=17; T3, n=21. Mann-Whitney test. n=number of cells, from 3 independent experiments.

Supplementary Figure 8. Early neurofilament accumulations in ALS iPSC-derived MNs.

(a, b) Representative images showing control and ALS iPSC-derived MNs labelled at T0 with antibodies against (a) the light neurofilament subunit NF-L or (b) the phosphorylated medium/high neurofilament subunits pNF-M/H. Images show both NF-L and pNF-M/H strong accumulations in MN cell bodies. Also, while neurite networks were similar both at intensity staining and density levels in control and ALS MNs stained for pNF-M/H, NF-L positive MN neurite networks were brighter with ALS MNs which appeared already slightly dashed. Comparisons of density neurite networks between T0 (here) and T1 (Figure 4b) assess of the reduced density networks of ALS MN cultures compared to control MNs. Scale bars: 10µm.

Supplementary Figure 9. Early alterations of the structural integrity of proximal axonal regions in SOD1 and C9ORF72 iPSC-derived MNs.

(a) panNav integrated density in the GAP. **(b, c)** GAP and AIS area. n = number of analyzed MNs from 3 independent experiments per lines. Controls (Ctrl33, Ctrl69-2): n=81. ALS without accumulation: SOD1 (SOD1-1, SOD1-2): n=74, C9ORF72 (C9ORF72-1, ²C9ORF72-2): n=71, TARDBP (TARDBP-1, TARDBP-2) n=71. ALS with accumulation: SOD1 (SOD1-1, SOD1-2): n=69, C9ORF72 (C9ORF72-1, ²C9ORF72-2): n=63. Kruskal-Wallis with Dunn's test (mean ± SEM). Statistical significance shown either between ALS and control MNs (*) or between MNs with or without accumulation (φ). ***p<0.001, ****/φφφφp<0.0001, φp<0.05.

Supplementary Figure 10. Abnormal protein accumulations in ALS iPSC-derived MNs.

(a) Control and SOD1^{N139D} MNs immune-stained with anti-SOD1 antibodies showing rare specific SOD1-positive dots in neurites of mutant β 3-Tubulin (TUJ1)-positive neurons at T1 (arrows) (n = 3 independent experiments). Scale bars: 10 μ m. **(b, c)** Control and TARDBP^{G348C} MNs co-stained with antibodies against β 3-Tubulin (TUJ1) and either (b) TDP43 or (c) phosphorylated TDP43 (pTDP43) showing nuclear TDP43 localization with rare nuclear TDP43 and cytoplasmic pTDP43 inclusions, only at T2. Arrows in insets indicate inclusions. (n = 3 independent experiments). Scale bars: 20 μ m.

| gene symbol | Gene name | Belongs to significant GO terms | Known functions | FC | Adjusted P-Value |
|--------------------|---|---|---|-----------|-------------------------|
| CHL1 | cell adhesion molecule L1 like protein | - Extracellular exosome - Proteinaceous extracellular matrix - Cell adhesion - Plasma membrane | -Recruits AnkG to the plasma membrane - cholinergic synapse formation | 362.8 | 2.75x10 ⁻⁶ |
| CTSF | Cathepsin F | - Extracellular space - Extracellular exosome | -Lysosomal enzyme -Role in Intracellular degradation and turnover of proteins | 357.0 | 1.17x10 ⁻³ |
| RIMBP2 | RIMS binding protein 2 | - Plasma membrane | Role in synaptic transmission | 29.5 | 1.41x10 ⁻³ |
| NNAT | neuronatin | | May function as a regulatory subunit of, ion channels | 876.5 | 3.77x10 ⁻³ |
| DACH1 | dachshund family transcription factor 1 | | Transcription factor | 4.2 | 4.36x10 ⁻³ |
| HSPA1L | heat shock protein family A (Hsp70) member 1 like | - Cell body | In conjunction with other hsp, this protein stabilizes existing proteins against aggregation and mediates the folding of newly translated proteins in the cytosol and in organelles | 3.9 | 6.71x10 ⁻³ |
| TCF4 | transcription factor 4 | | Transcription factor | 3.7 | 6.71x10 ⁻³ |

Table 1. Genes upregulated in ALS mMN vs control mMN.

Supplementary Table 1. ALS patients, control subjects and iPSC clones.

| | | | | | |
|------------------------|-------|----|-------------|-----------|--|
| SOD1-1 | | | | | |
| SOD1-2 | woman | 50 | Lower limbs | >25 years | |
| SOD1-3 | | | | | |
| SOD1-ISO | | | | | |
| ¹ C9ORF72-1 | man | 70 | Lower limbs | 3 years | |
| ² C9ORF72-2 | | 51 | | | |
| ² C9ORF72-3 | man | | Bulbar | 3 years | |
| TARDBP-1 | | | | | |
| TARDBP-2 | man | 53 | Lower limbs | 6 years | |
| TARDBP-3 | | | | | |
| TARDBP-ISO | | | | | |
| Ctrl11 | man | 11 | - | - | |
| Ctrl33 | man | 33 | - | - | |
| Ctrl60 | man | 60 | - | - | |
| Ctrl69-1 | | | | | |
| Ctrl69-2 | woman | 69 | - | - | |

ISO = isogenic control iPSC clone generated with the CRISPR/CAS9 technology.

(Supplementary Table 2: excel file)

Supplementary Table 3: Description of ALS patients (*post-mortem* tissues)

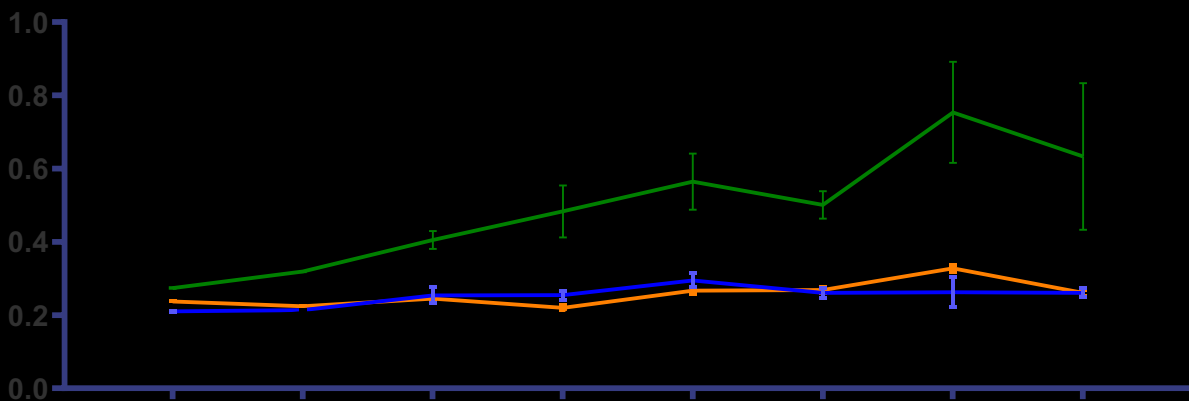
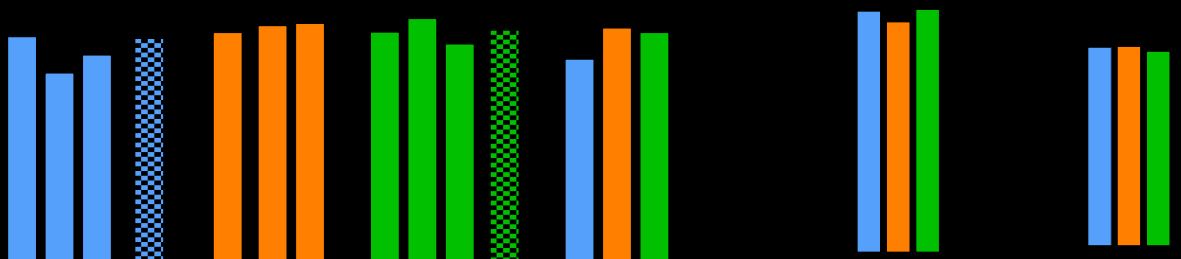
| | | | | |
|--|------|------------|----|------------------------|
| | male | Upper limb | 73 | Aggressive (<1year) |
| | male | Bulbar | 55 | Aggressive (1,6 years) |
| | male | Upper limb | 47 | Aggressive (3 years) |

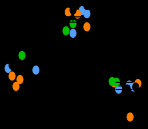
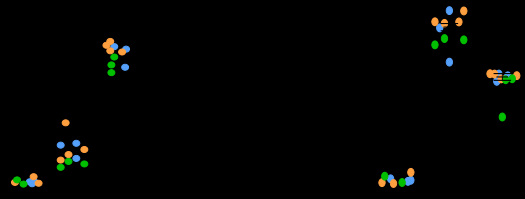
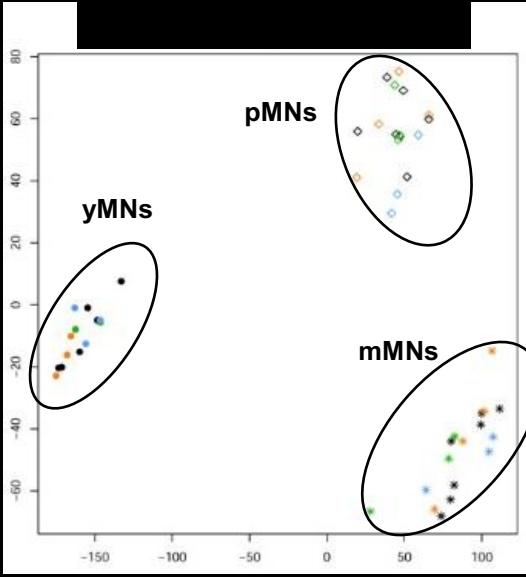
Supplementary Table 4: Primary antibodies

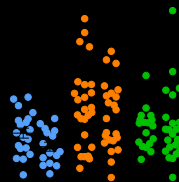
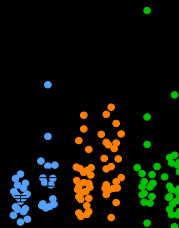
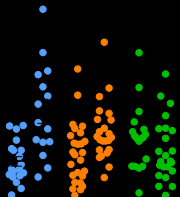
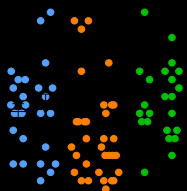
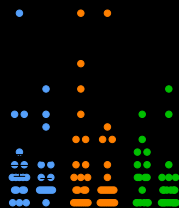
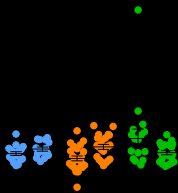
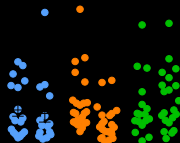
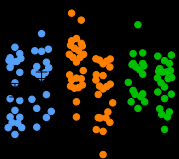
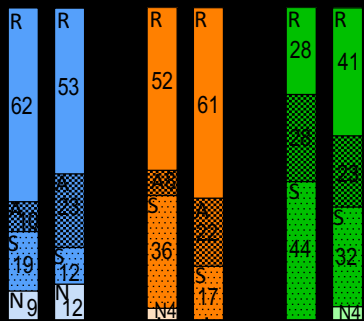
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|--|--------|--------|-----------------|
| | IgG2a | 1/200 | Chemicon |
| | IgG2a | 1/200 | Neuromabs |
| | Rabbit | 1/50 | Sigma |
| | Rabbit | 1/100 | Abcam |
| | IgG1 | 1/500 | Chemicon |
| | Rabbit | 1/100 | Abcam |
| | Rabbit | 1/500 | Millipore |
| | Igg1 | 1/200 | Sigma |
| | Rabbit | 1/1000 | Millipore |
| | Rabbit | 1/200 | Alomone Labs |
| | Igg1 | 1/300 | Millipore |
| | IgG1 | 1/3000 | CosmoBio |
| | IgG2a | 1/200 | Chemicon |
| | Igg1 | 1:4000 | Biolegend |
| | IgG3 | 1/100 | Chemicon |
| | Rabbit | 1/100 | Proteintech |
| | IgGM | 1/100 | Chemicon |
| | Igg2a | 1/500 | Covance |
| | Rabbit | 1/1000 | Synaptic System |

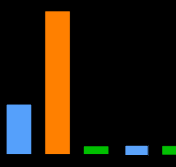
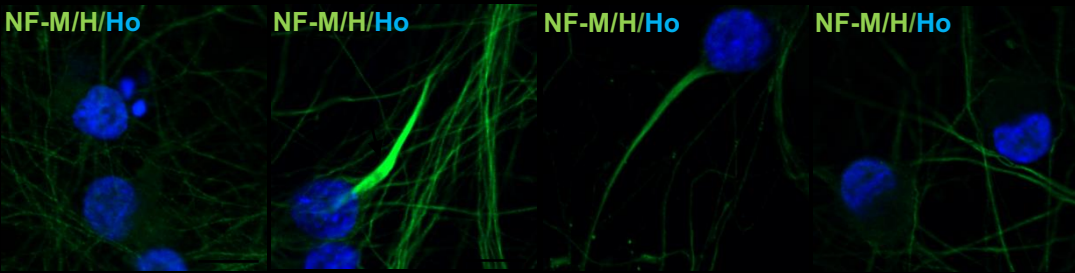
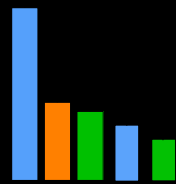
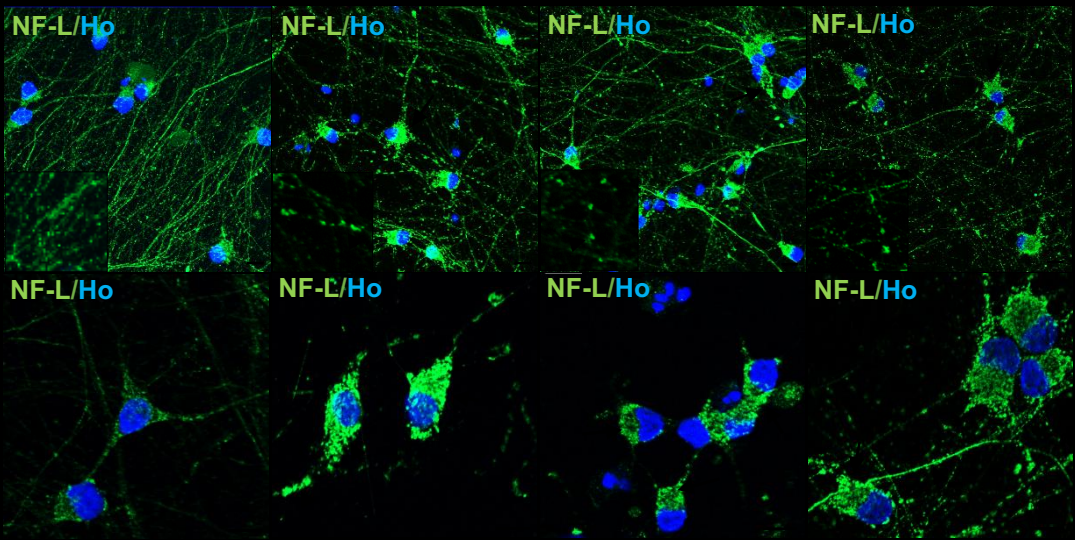
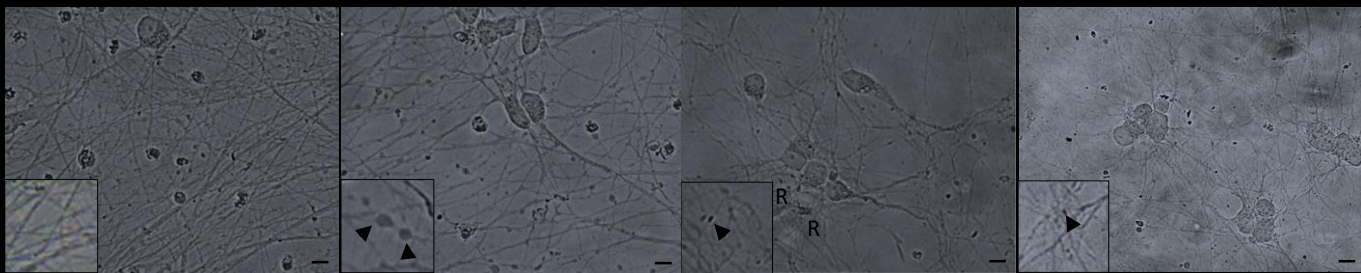
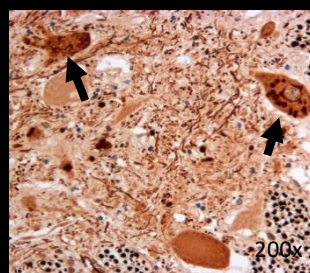
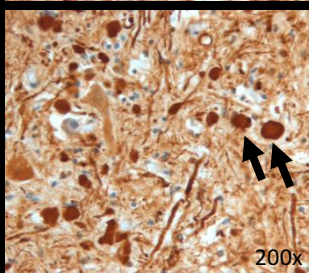
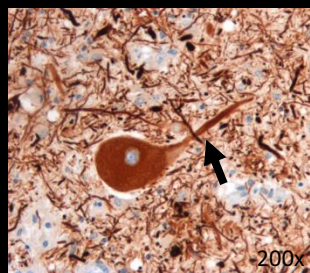
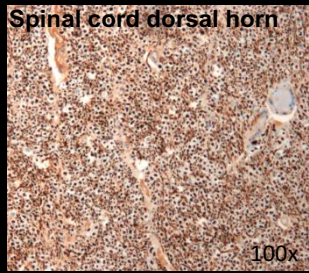
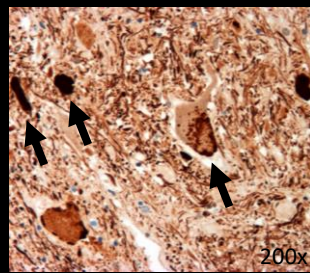
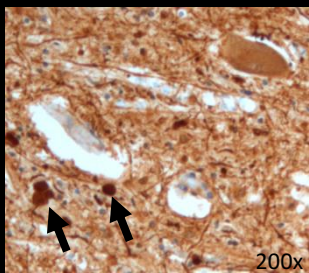
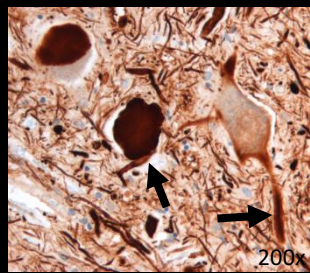
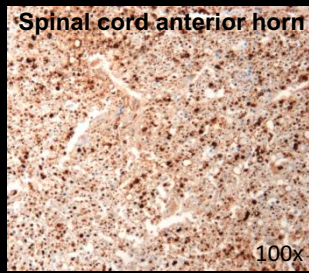
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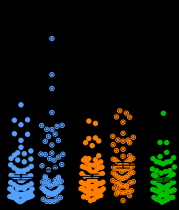
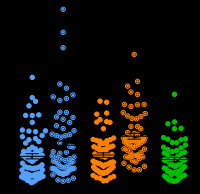
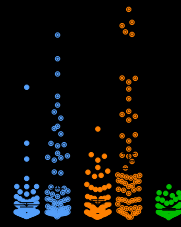
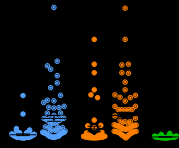
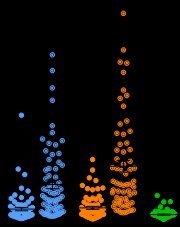
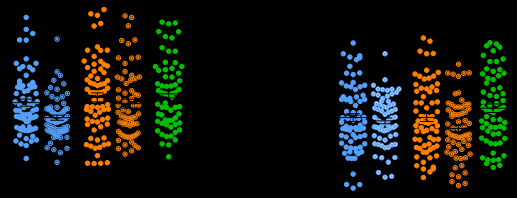
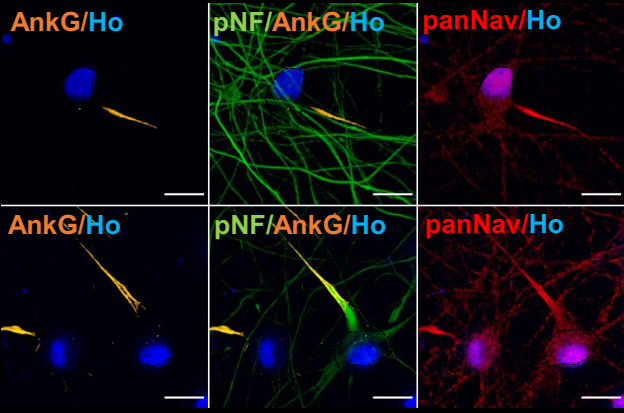
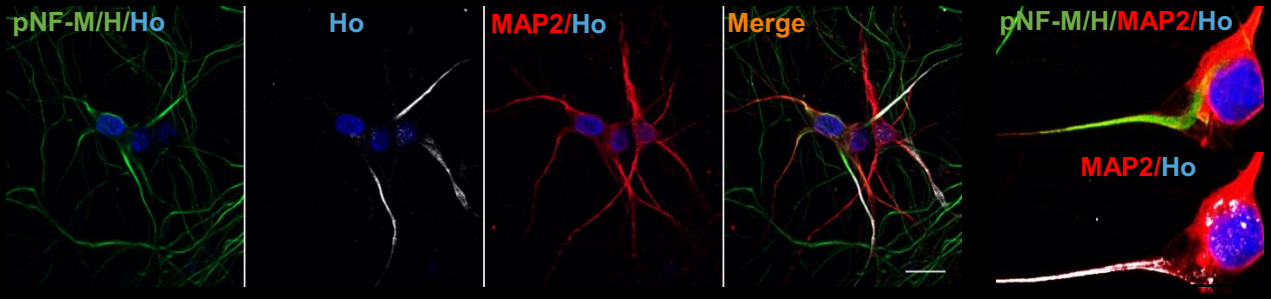
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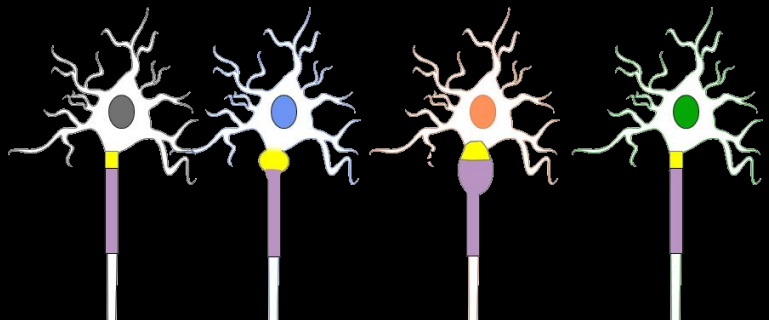
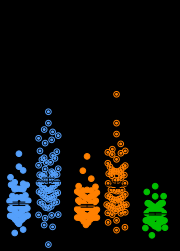
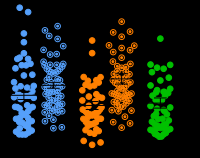
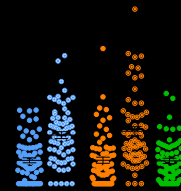


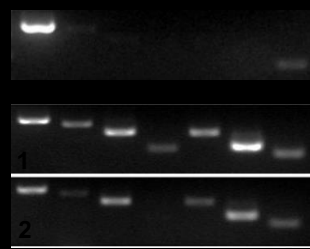
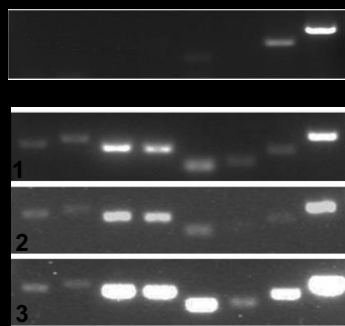
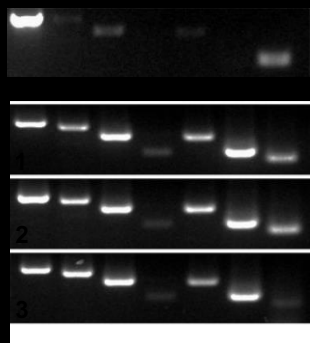
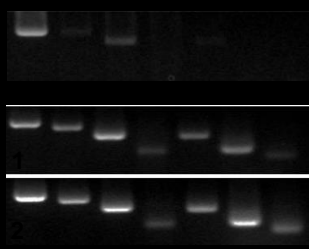
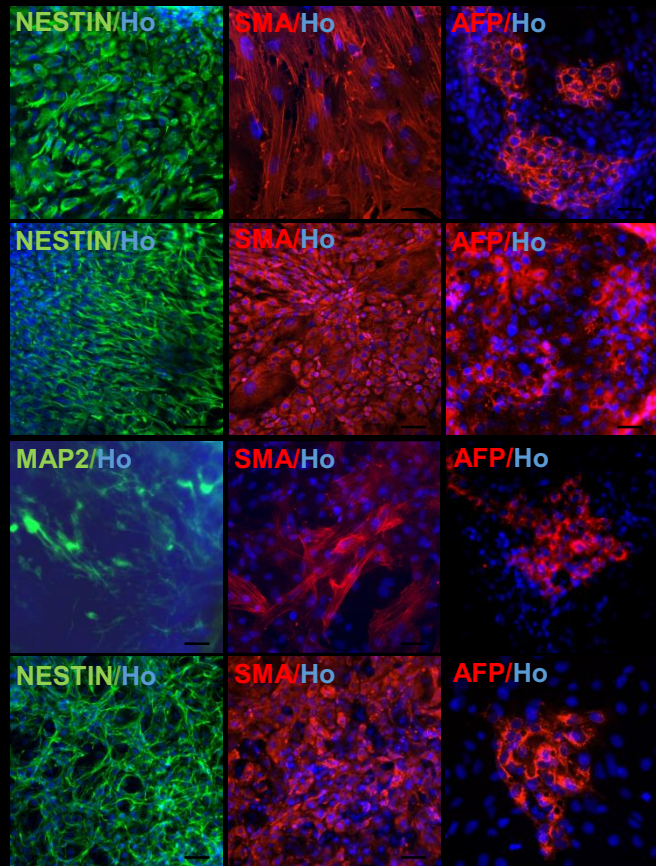
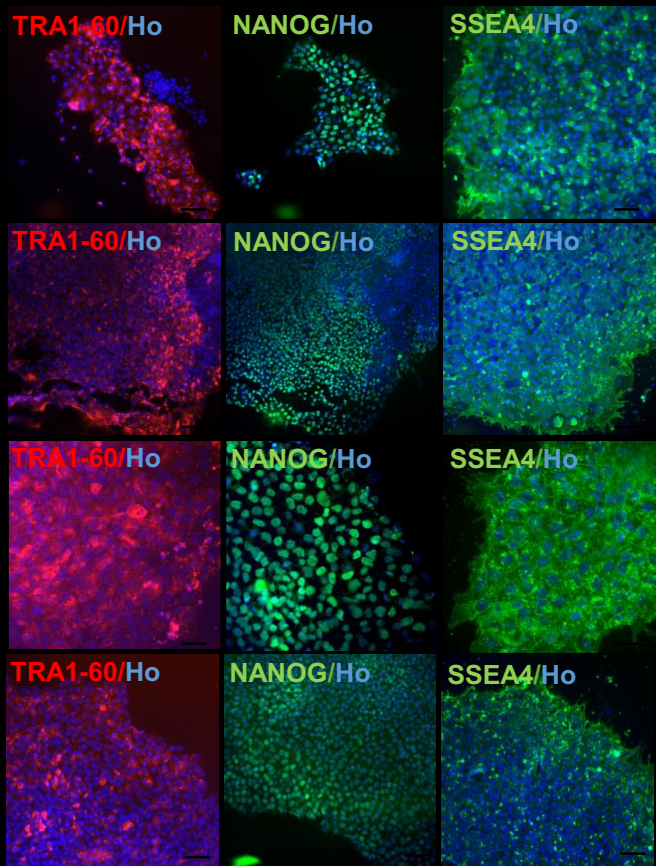


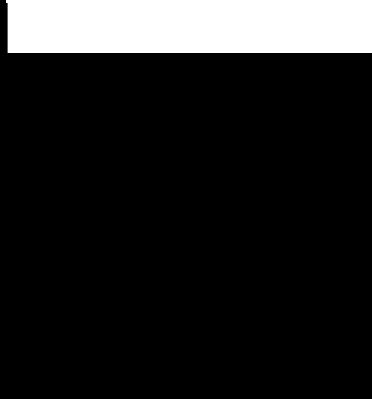
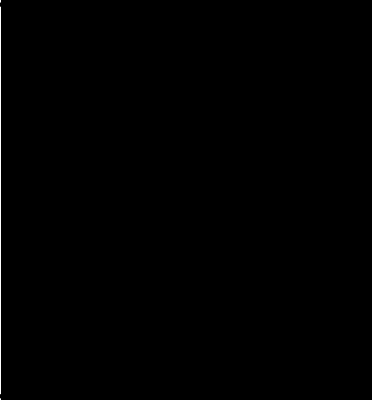
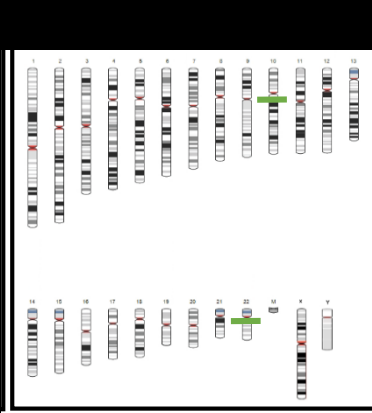
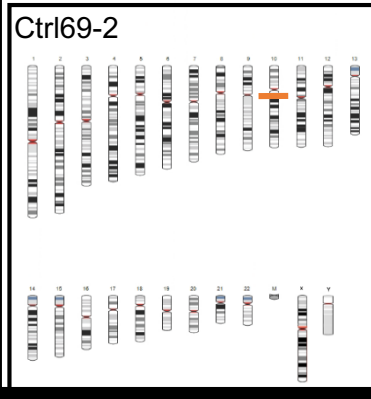
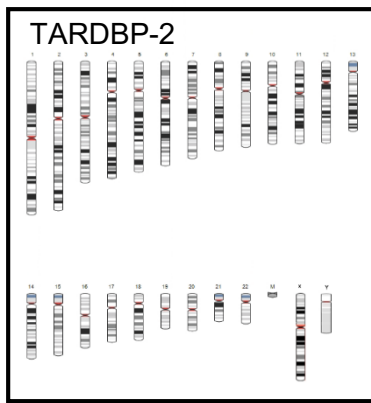
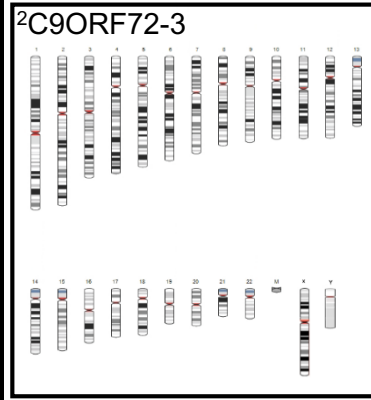
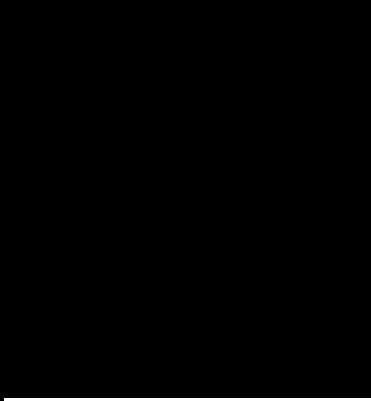
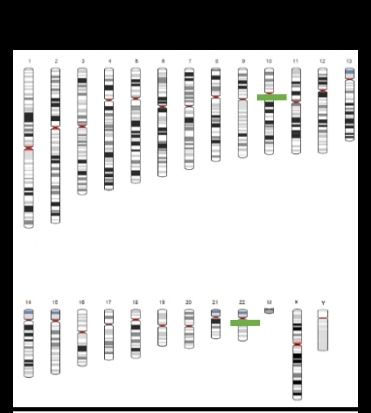
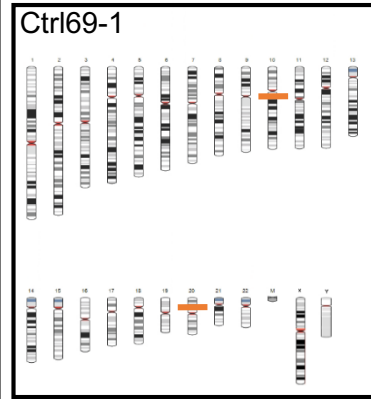
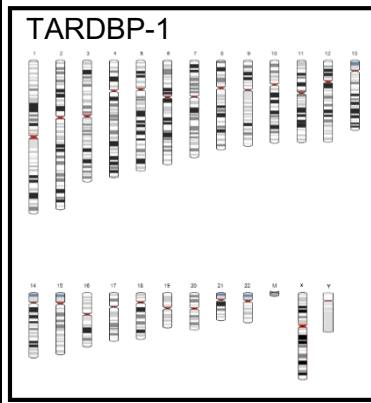
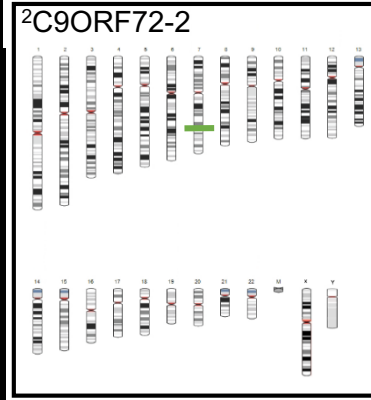
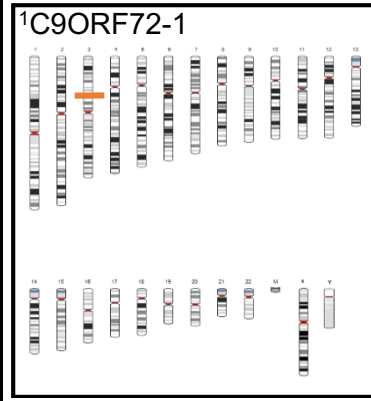
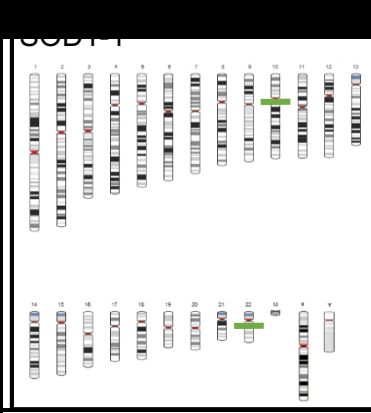
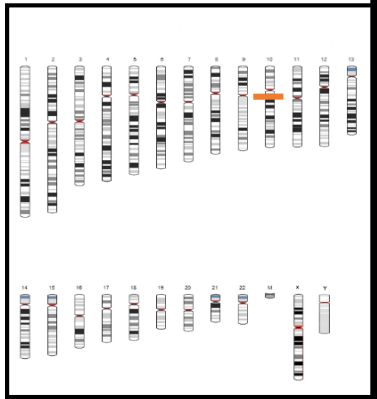
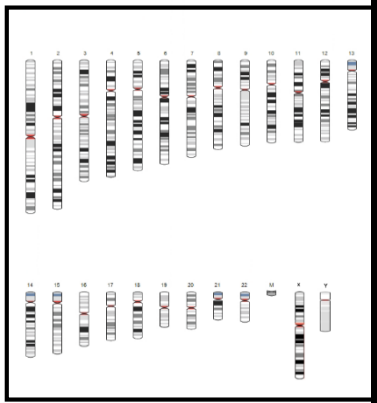
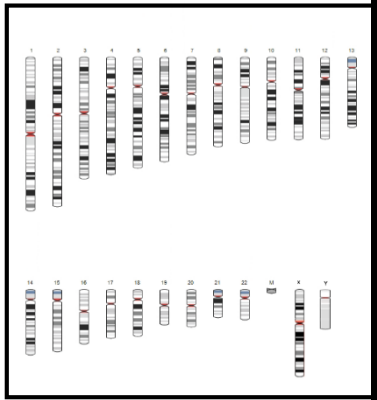
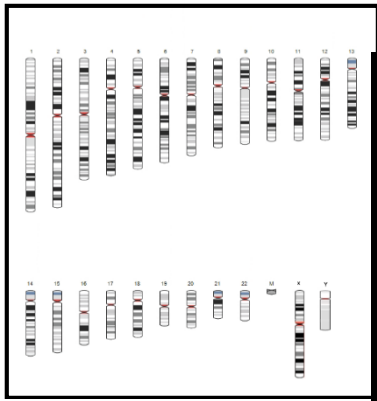
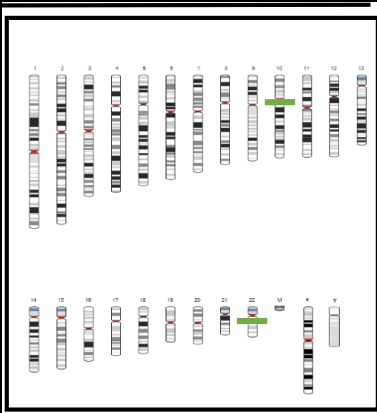




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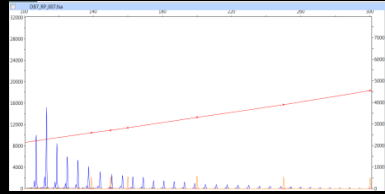
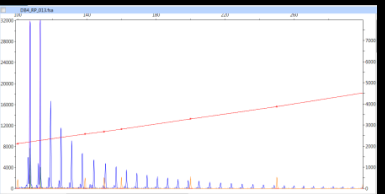
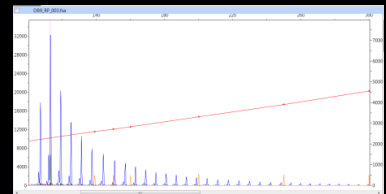
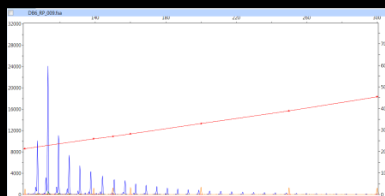
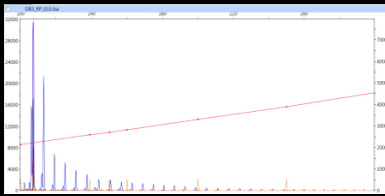
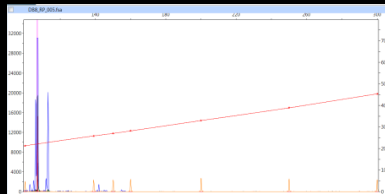
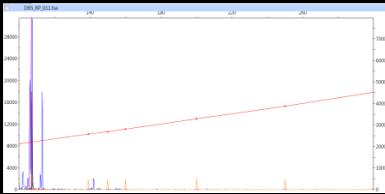
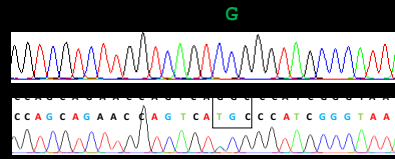
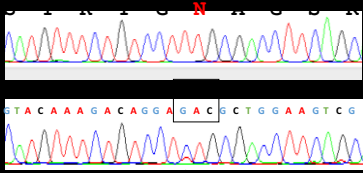
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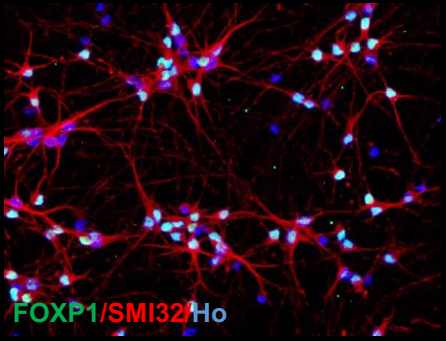
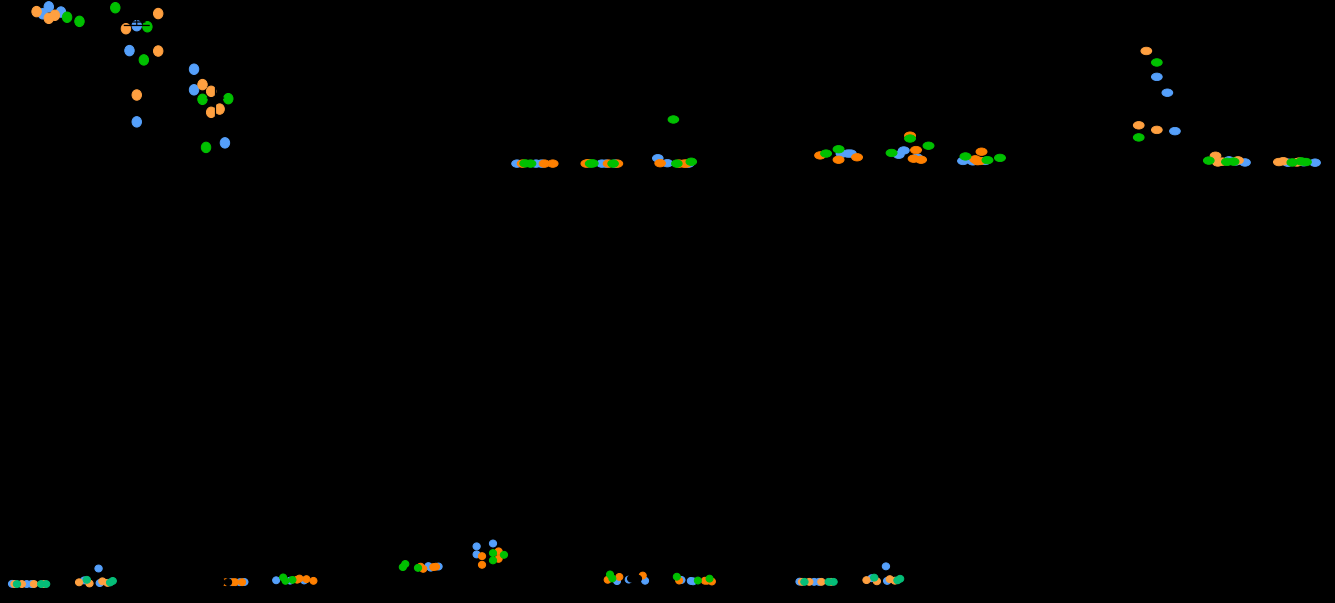
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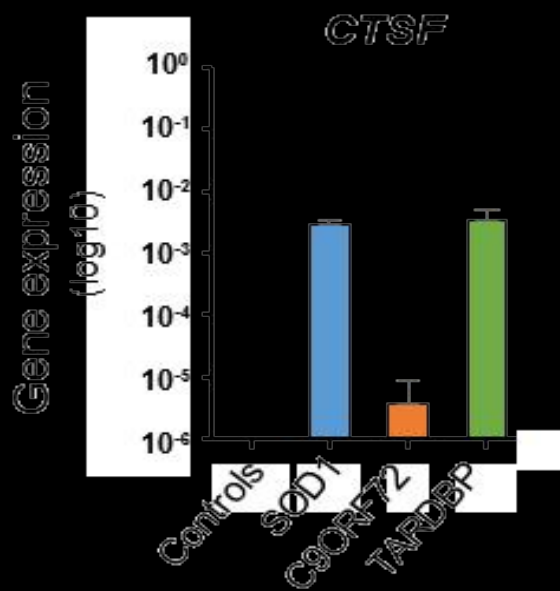
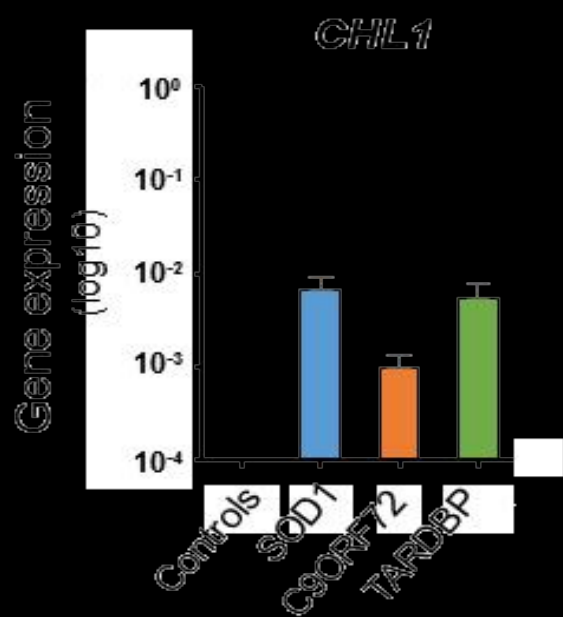
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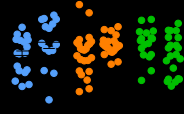
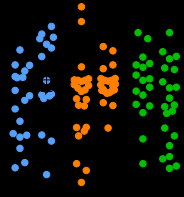
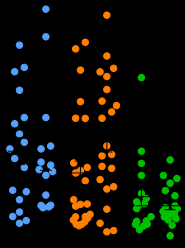
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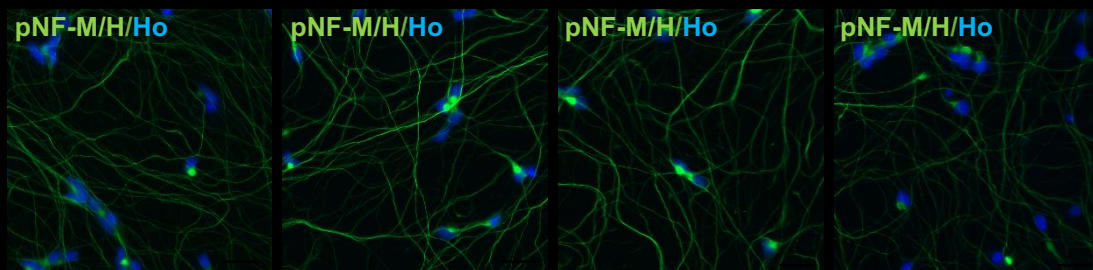
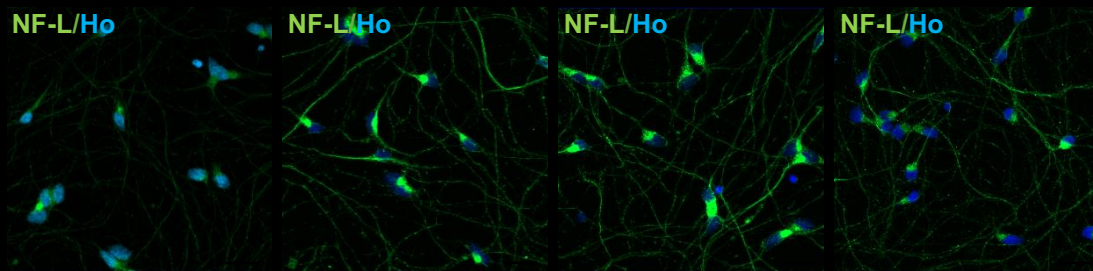
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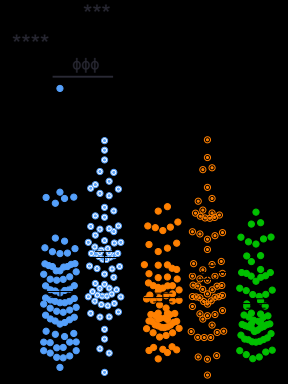
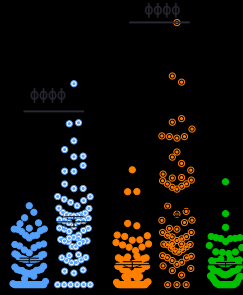
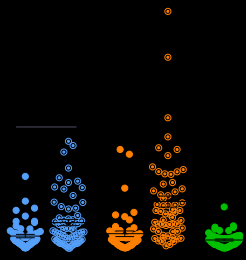


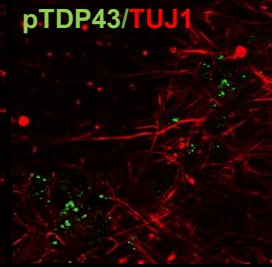
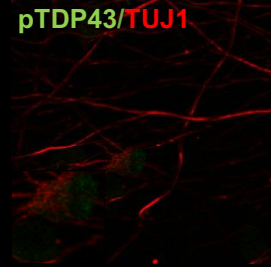
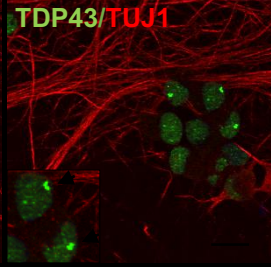
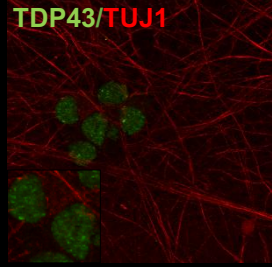
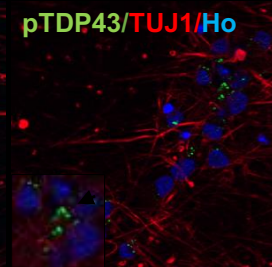
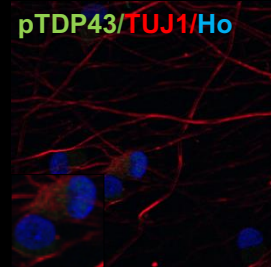
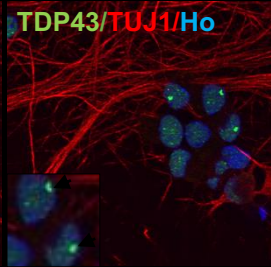
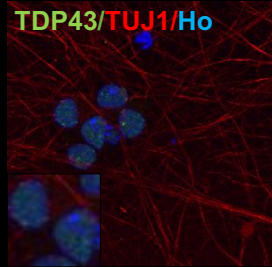
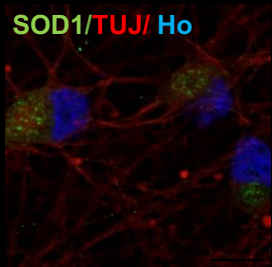












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Deciphering the respective contribution of macrophages and microglia on motor neuron degeneration in Amyotrophic Lateral Sclerosis using Induced pluripotent stem cells

Amyotrophic lateral sclerosis (ALS) is a rapidly fatal progressive neurodegenerative disease affecting motor neurons (MN). Despite the discovery of several genetic causes, numerous mechanisms involved in MN degeneration in different forms of ALS as well as a large number of therapeutic trials based on these results, there is to date no curative treatment. There is therefore an urgent need to find new therapeutic avenues. One of the reasons for the failure of the trials is the great heterogeneity of the disease. Although neuroinflammation is a common signature of all ALS patients, its mechanisms, contribution and timing, appear to differ depending on the form of the disease. The team is therefore interested in understanding its role in the different forms of ALS, with a particular interest in innate immune cells. Indeed, macrophages and microglia are known to be activated in all ALS patients and have recently been shown to participate differently in the pathology in the SOD1 mouse model. However, what about in other ALS forms? In order to answer this question, my thesis project consisted in using human induced pluripotent stem cells (iPSC) to generate MNs, macrophages and microglia, in order to analyse and compare the trophic and toxic roles of innate immune cells in MN degeneration. My results show that the two ALS cell types respond differently to MN degeneration compared to control cells, and exhibit specific defects of the endolysosomal pathway and specific inflammatory secretory profile over time. Taken together, my results identify deregulations common and distinct between the different ALS forms, which could represent interesting targets for future therapies. More broadly, the identification of these deregulations distinct to the subtypes of patients, could open up new perspectives to better identify and classify ALS patients in order to offer more personalised therapies.

Identification du rôle respectif des macrophages et des cellules microgliales envers la dégénérescence des motoneurones dans la Sclérose Latérale Amyotrophique en utilisant les cellules souches pluripotentes induites humaines

La sclérose latérale amyotrophique (SLA) est une maladie neurodégénérative progressive rapidement mortelle affectant les motoneurones (MN). Malgré la découverte de plusieurs causes génétiques, de nombreux mécanismes impliqués dans la dégénérescence MN dans différentes formes de SLA ainsi que d'un nombre élevé d'essais thérapeutiques basés sur ces résultats, il n'existe à ce jour aucun traitement curatif. Il y a donc un besoin urgent de trouver de nouvelles voies thérapeutiques. L'une des raisons de l'échec des essais est la grande hétérogénéité de la maladie. Bien que la neuroinflammation soit une signature commune de tous les patients SLA, ses mécanismes, sa contribution et sa temporalité semblent différer selon les formes de la maladie. L'équipe s'intéresse donc à comprendre qu'elle est son rôle dans les différentes formes de SLA, avec un intérêt particulier pour les cellules immunitaires innées. En effet, les macrophages et la microglie sont connus pour être activés chez tous les patients SLA et ont récemment montré qu'ils participaient différemment à la pathologie dans le modèle murin SOD1. Mais qu'en est-il des autres formes de SLA ainsi que chez les patients? Afin de répondre à cette question, mon projet de thèse consistait à utiliser des cellules souches pluripotentes induites humaines (iPSC) pour générer des MNs, des macrophages et des microglies, afin d'analyser et comparer les rôles trophiques et toxiques des cellules immunitaires innées dans la dégénérescence MN. Mes résultats montrent que les deux types cellulaires SLA réagissent différemment à la dégénérescence MN par rapport aux cellules témoins, et présentent des défauts spécifiques de la voie endolysosomale et divers profils dynamiques de sécrétion inflammatoire au fil du temps. Mes résultats identifient des dérégulations communes et distinctes aux différents types de SLA, qui pourraient représenter des cibles intéressantes pour de futures thérapies. Plus largement l'identification de ces dérégulations distinctes entre sous types de patients, pourraient ouvrir de nouvelles perspectives de possibilités thérapeutiques plus phénotype ciblé.